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CYANIDE-INSENSITIVE RESPIRATION IN PEA COTYLEDONS

by

© TERRANCE WAYNE JAMES

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Cyanide-Insensitive Respiration in Pea Cotyledons submitted by Terrance Wayne James in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Cyanide-resistant respiration in the cotyledons of germinating peas has been studied using isolated mitochondria. The mitochondria were isolated on a sucrose-step gradient by zonal centrifugation. Up to 15% of the succinate oxidation was resistant to 0.2 mM KCN. A greater proportion of the respiration was resistant to cyanide when α-ketoglutarate or malate was used as the respiratory substrate.

Experiments were conducted to determine how the pathway interacted with the electron transport chain. The results support the involvement of ubiquinone in the cyanide-resistant pathway and indicate that branching to the alternate pathway may take place at that point. Duroquinol was observed not to donate electrons to the alternate pathway and a new inhibitor of cyanide-resistant respiration, chloroquine, was found.

Some factors affecting the amount of cyanide-resistant respiration were also investigated. The age of the cotyledons was found to be a very important factor. Mitochondria isolated from cotyledons during the first 24 hours of germination had virtually no cyanide-resistant respiration, but thereafter cyanide resistance gradually increased until the sixth or seventh day of germination. The presence of light during germination, or the inclusion of chloramphenicol or azide in the germination medium did not influence the amount of cyanide-resistant respiration. However, the plant hormone ethylene was found to affect the amount of cyanide resistance. Cotyledons germinated in an atmosphere containing approximately 100 ppm ethylene were found to have greater amounts of cyanide-resistant germination than when ethylene was absent.

INTRODUCTION

The past century has seen a steady increase in the productivity of plants and domestic animals. Much of this gain in productivity has come as a result of improved agronomic practices and the exploitation of existing genetic potentials by plant and animal breeders. There are however, indications that increases achieved by these technologies are plateauing and that a new generation of agricultural research is required.

Dr. S. H. Wittwer has pointed out that this new generation of agricultural research "depends on mission-oriented basic research relating to the biological processes that control and now limit crop and livestock productivity."[1] The underlying hope is that these processes may be manipulated "to enable plants and animals to more effectively utilize environmental resources."[2]

Cyanide-resistant respiration may prove to be an area where such manipulation is possible. Many authors have pointed out that cyanide-resistant respiration does not seem to be as efficient in the energy economy of the cell as other respiratory pathways. There have even been some preliminary attempts to produce plants that would genetically lack cyanide-resistant respiration. However such research is probably premature since we know so little about the nature and composition of the cyanide-resistant respiratory pathway, and of its importance to the plant. The studies reported herein were directed at furthering our understanding of this important biological process with the hope that they may in some way contribute to the long range goal of increasing agricultural productivity.

[1] From S.H. Wittwer (113)
[2] Ibid

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ABBREVIATIONS

ADP	-	adenosine diphosphate
AMP	-	adenosine monophosphate
ATP	-	adenosine triphosphate
CAM	-	crassulacean acid metabolism
CCP	-	carbonyl cyanide m-chlorophenylhydrazone
epr	-	electron paramagnetic resonance
NADH	-	nicotinamide adenine dinucleotide (reduced form)
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced form)
m-CLAM	-	m-chlorobenzhydroxamic acid
RCR	-	respiratory control ratio
SHAM	-	salicylhydroxamic acid
Tes	-	N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid
TMPD	-	N,N,N',N',-tetramethyl-p-phenylamine-diamine
Tris	-	tris (hydroxymethyl) aminoethane

I. LITERATURE REVIEW

A. Historical Developments in the Study of Cyanide-Resistant Respiration

The poisonous properties of hydrogen cyanide, hydrocyanic acid, or prussic acid, names that all refer to the same compound, have been known for nearly 200 years. Fontana pointed out in an early book on venoms and poisons that the leaves of the "cherry laurel" (Prunus lauocerasus) contained "a poison which for some years has been celebrated in Europe." Fontana (33) observed that the poison "exerted a violent effect on animals" and killed them rapidly when it was administrated by injection, by mouth, or when it was merely kept in contact with the mucous membranes of the mouth.

Hydrogen cyanide has given biologists a research tool which has proven very useful in the unravelling of the mechanisms of biological oxidations. Keilin (57) acknowledges this in his classic book on the history of the study of respiration and discusses some of the experiments that elucidated the mechanism by which hydrogen cyanide exerts its poisonous action.

The first clue came in 1857 when Claude Bernard (9) found that the blood in animals poisoned by cyanide remained red on both sides of the heart. Bernard knew that this was not a direct effect of cyanide on the blood, but it remained for Hoppe Seyler to interpret his results. Hoppe Seyler (47) correctly deduced that the action of cyanide was to inhibit the utilization of oxygen by the tissues. The reason why cyanide inhibited the utilization of oxygen however, could not be ascertained until the experiments of Keilin (56).

Keilin's experiments on cytochrome established the seemingly universal occurrence of the cytochromes a, b, and c, their reversible oxidation and reduction, and their role in cellular respiration. As well he was able to localize the effects of some respiratory inhibitors, including cyanide, because of their action on the cytochrome system. In later experiments Keilin and Hartree (58) were able to show that cyanide combined with cytrochrome a_3 to form an "a_3 . . . CN complex that was no longer able to undergo reversible oxidation and reduction.

However even while Keilin was carrying out these very important experiments with cytochrome and with cyanide, other workers were reporting that not all respiration was, in fact, inhibited by cyanide. In 1918 Warburg (106) reported that the respiration of starved yeast was not affected by cyanide, and in 1927 Emmerson (31) reported that the respiration of Chlorella protothecoides could in fact be accelerated by cyanide under some conditions. This occurred when HCN (10^{-4}M) was added without exogenous substrate to cells that had been grown autotrophically in an inorganic medium.

Another instance in which cyanide stimulated respiration was reported by Hanes and Barker in 1931 (42). Their experiments utilized whole potato tubers that had previously been stored for some months at 15°C. Under these conditions the potatoes had a steady but very low respiration rate. When a stream of air containing small amounts of cyanide was passed over these tubers both the oxygen consumption and the CO_2 output began to increase dramatically. The increase in respiration began about 24 hours after the initial exposure to cyanide, but the maximum rate was not reached until 10 or 15 days later. Higher concentrations of cyanide produced a proportionally greater response, at least

within the concentration range tested (0.14 cc HCN/liter to 1.45 cc HCN/liter). The respiratory quotient remained near 1 in all cases, except at the very highest level of cyanide tested. The effects were reversible in that removing the potatoes from the cyanide-containing atmosphere before the completion of the experiment caused the respiration rate to fall before it achieved its maximum.

These experimental results proved very difficult to explain. The authors felt that cyanide perhaps had an activating effect on the amylases of the potato and that the increase in respiration could be explained by an increase in the soluble sugar content of the potato. They had a limited amount of experimental evidence to support their opinion, but their experiments did not receive a great deal of attention and further experiments on this phenomenon were not carried out for the next thirty years.

Another early study on cyanide insensitive respiration that deserves mention is one that was done by Genevois in 1929 (25). He observed that etiolated Lathryus seedlings had a portion of their respiration resistant to cyanide. As the seedlings aged the total respiration rate fell, but the cyanide resistant respiration rate did not.

The peculiar respiration of the Arum family and its relationship to cyanide insensitive respiration was studied by Van Herk in 1937 (102). It had been discovered as early as 1778 (62) that some members of the Arum family produced heat when they flowered. Van Herk extended these studies and found that the heat producing organ in Sauromatum, a sterile appendix, had a greatly accelerated respiration rate. Moreover, the oxygen uptake of this organ was not inhibited by cyanide. Since that time members of the Arum family have been used extensively in the study

of cyanide-resistant respiration.

Van Herk (103) studied the mechanism of cyanide-resistant respiration further. He was unable to detect cytochrome or cytochrome oxidase in the Sauromatum appendix. He did however, isolate an autoxidizable flavoprotein and he ascribed the cyanide insensitivity to this enzyme.

A major step in the study of cyanide resistant respiration occurred in 1955. James and Elliot (51) used the recently worked out technique of differential centrifugation to isolate mitochondria from the spadix of Arum mataculatum. The respiration of these mitochondria proved to be relatively insensitive to cyanide.

One year later Bendall and Hill (8) disproved the earlier suggestion by Van Herk that the Arum spadix did not contain cytochrome and started workers off on a path of research that eventually proved unfruitful. Using the microspectroscope, the instrument with which Keilin had discovered cytochrome, they found that mitochondria isolated from the spadix of Arum mataculatum had absorption bands at 605 nm, 560 nm and 550 nm. This corresponded roughly to the cytochromes a, b, and c from other tissues. However, the b band seemed to be unique in that it remained oxidized in the presence of succinate and cyanide even though the a and c bands were readily reduced. The authors called this cytochrome b_7 and ascribed the cyanide insensitivity of the Arum family to its presence. This view became widely accepted and can still be found in some relatively recent textbooks (39). Storey and Bahr (96) were the first to question this view. They found that cytochromes of Symplocarpus foetidus were nearly fully reduced in the presence of antimycin and yet the cyanide resistant pathway continued to operate under these conditions. and Bonner (7) also found this to be true. The view that cytochrome b_7

was involved in cyanide-resistant respiration persisted however. The discovery (27) that mitochondria from Arum mataculatum, purified on a sucrose gradient, did not contain cytochrome b_7 finally seemed to dispell the view that cytochrome b_7 was involved in cyanide resistant respiration.

An alternative suggestion concerning the mechanism of cyanide resistant respiration appeared shortly after the discoveries by Bendall and Hill. It was called the excess-oxidase theory and was carefully examined by Chance and Hackett (22), Yocum and Hackett (115), and Hackett and Haas (37). The following two points provide the basis for this theory: 1) Plant mitochondria contained cytochrome oxidase in much larger quantities than the other respiratory components, and 2) Even in the presence of cyanide a small amount of oxidized cytochrome a_3 could be found. It was thought that a slow turnover of the cyanide-cytrochrome a_3 complex could account for the cyanide insensitive respiration.

However Wiskich and Bonner (110) and Bendall and Bonner (7) were quick to refute this theory. In their experiments they used a mixture of ascorbate and TMPD as the respiratory substrate, which donates electrons to the respiratory chain after the second site of phosphorylation, probably at the level of cytrochrome c. Oxidation of this substrate by isolated sweet potato mitochondria was very rapid and was practically 100% inhibited by cyanide. The authors felt that the small amount of residual respiration could reflect a slow turnover of the inhibited form of cytochrome a_3, or perhaps a small amount of autoxidation of the substrate. When succinate was added to these inhibited mitochondria the respiration rate increased dramatically. It was clear that the excess oxidase theory could not account for these results and

it became necessary to postulate that a second oxidase was indeed present in the sweet potato mitochondria.

In summing up knowledge to 1971, Bonner and Bendall (7) concluded that there was little positive information to characterize the alternative pathway. However, they felt that an earlier suggestion by Okunuki (76) (first proposed in 1939) came the closest to fitting all the experimental results. As a result of his studies on pollen respiration, Okunuki proposed a branched respiratory chain, the alternative pathway connecting with the cytochrome pathway on the substrate side of cytochrome c, prior to the antimycin block. More recent studies have upheld this view.

B. The Electron Transport Chain of Plant Mitochondria

1. General Features of Electron Transport in Plant Mitochondria

The respiratory chain of plant mitochondria as outlined by Palmer (77) is shown in Figure 1. In many ways it resembles that of mammalian mitochondria. The soluble Krebs cycle enzymes present in the matrix pass electrons to NADH and from there the electrons are passed on to flavoproteins, then to iron sulfur proteins, to ubiquinone, and finally to the cytochromes. There are three phosphorylation sites analogous to those in mammalian mitochrondria. When succinate is the respiratory substrate there are only two phosphorylation sites and the succinate dehydrogenase is membrane bound.

Much information still needs to be gathered about the iron-sulfur proteins and the flavoproteins in plant mitochrondria (15). Some appear to have mammalian counterparts; others do not.

The c and a type cytochromes closely resemble those of mammalian mitochondria (49). The only differences would appear to be slight

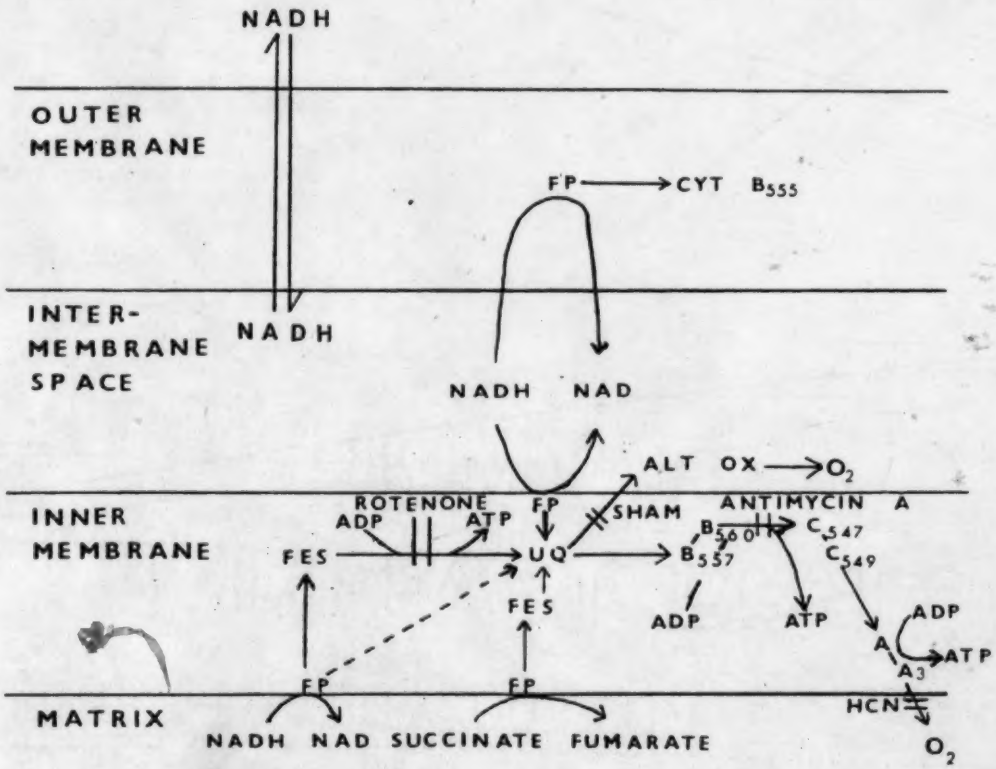


FIGURE 1. The electron transport chain of plant mitochondria.

shifts in the absorbance peaks. For example, the α-absorption peak of cytochrome a occurs between 601 nm and 603 nm rather than the 605 nm of beef heart mitochondria cytochrome a. As in other mitochondria the identities and behavior of the b type cytochromes are not well understood. Plant mitochondria may contain as many as 4 b cytochromes reducible by natural donors (77). The proton motive ubiquinone cycle proposed by Mitchell (73) and applied to plant mitochondria by Rich and Moore (87) may help to clarify the role of the b cytochromes.

The ubiquinone in plant mitochondria is ubiquinone 10 (10). Ubiquinone seems to act as a collecting agent for reducing equivalents prior to the second site of phosphorylation (76). It may also serve as the branch point for the cyanide resistant pathway (18, 48).

2. Oxidation of NADH by Plant Mitochondria

Plant mitochondria contain a minimum of 4 different NADH dehydrogenases (77). However, one is associated with the outer membrane and does not normally transfer reducing equivalents to the mitochondrial phosphorylating electron transport chain (28). It is distinguished by the fact that it is specific for the 4-α-hydrogen of the NADH whereas the other dehydrogenases are specific for the 4-β-hydrogen.

A second of these plant NADH dehydrogenases is associated with the outer surface of the inner membrane (28, 78). It allows the plant mitochondria to oxidize externally added NADH. Plant mitochondria oxidize externally added NADH rapidly and with an ADP:O ratio of 2.0. This fact and the fact that the oxidation of exogenous NADH is sensitive to antimycin indicates the electrons from the NADH are added between sites 1 and 2 of the main electron transport chain. That is, electrons are

transferred either to ubiquinone or directly to the cytochrome b region.

One of the striking differences between plant and animal mitochrondria is their sensitivity to the inhibitor rotenone. In animals rotenone is one of the most potent of the respiratory poisons. The same is not true for plants (50, 111). Possible reasons for the difference include structural differences in the iron-sulfur proteins to which rotenone binds, or differences in the lipid environment surrounding these proteins, preventing binding of the inhibitor. Another very plausible reason and one strongly espoused by Palmer (19, 77) is that there are two internal NADH dehydrogenases, one sensitive to rotenone and coupled to ATP synthesis and one resistant to rotenone and not coupled to ATP synthesis. A strong piece of evidence for this hypothesis is the fact that rotenone causes the ADP:O ratios to fall by approximately 1 unit when either malate or α-ketoglutarate is the respiratory substrate (19, 111).

Plant mitochondria also possess malic enzyme in addition to malate dehydrogenase. This can be shown by kinetic analysis for malate oxidation by whole mitochondria. Both Brunton and Palmer (19) and Wedding, Black, and Pap (109) have found bimodal substrate saturation curves for malate. Wedding et al attribute the high affinity part of the curve to malate dehydrogenase, and the low affinity part to malic enzyme. Brunton and Palmer have the reverse interpretation. Other workers (68) have demonstrated the presence of malic enzyme by isolating it from purified sonicated mitochondria, and by showing that pyruvate and not oxaloacetate is often a product of malate oxidation.

Piericidin A (an inhibitor that probably acts at the same site as rotenone (52)) has been observed to have differing inhibition patterns depending on the concentration of malate used in the assay. At high

malate concentrations (60 mM), a condition which Brunton and Palmer (19) claim favors malate dehydrogenase, they found that Piericidin A caused only a small inhibition in oxygen uptake, similar to the inhibition observed when α-ketoglutarate was the substrate. However when conditions favored the operation of malic enzyme (10 mM Malate), a large transient inhibition of oxygen uptake occurred, and after this the recovered rate approximated the inhibited rate of α-ketoglutarate oxidation. Their interpretation of these experiments is that malic enzyme has preferential access to the phosphorylating rotenone-sensitive pathway. Palmer refers to this as kinetic compartmentation (77).

Palmer has done other experiments using oxalacetate as an inhibitor of malate oxidation to support contentions concerning the two NADH dehydrogenases and kinetic compartmentalization (79). However the conclusions from his experiments are by no means clear cut and there are those that disagree with them. Wiskich and Rayner (111) acknowledge the existence of the rotenone insensitivity, but feel it might simply be a short circuit -- an artifact of mitochondrial isolation, and may have little physiological significance.

3. The Cyanide Resistant Pathway

a. Organization of the Alternate Pathway

Another major difference between plant and mammalian mitochondria is that the former possess an alternate cyanide-resistant terminal oxidase. This pathway is quite widespread among the plant and microbial kingdoms, and has been reported in lower animals as well (45). When present, the cyanide resistant pathway does not replace the cytochrome pathway, but rather co-exists with it.

Questions concerning the arrangement of the two pathways and of the apportionment of electons between the two pathways then arise. Henry and Nyns (45) have discussed four different ways in which the two pathways could be arranged. The first case has the two pathways connecting only at the substrate level. They have common substrates but separate substrate dehydrogenases and separate electron transport chains. In the second case the two pathways share substrate dehydrogenases, but no member of the electron transport chain is common to both pathways. The third and most commonly accepted arrangement is that the first part of the electron transport chain is common to both pathways and branching occurs at a single point. It is also possible to envision branching occurring at more than one point, which is the fourth possibility discussed by Henry and Nyns.

Bahr and Bonner (3) attempted to find direct experimental evidence for the third hypothesis. It was their feeling that if the flavoprotein dehydrogenases had equal access to both pathways, then the flavoprotein complement of the mitochondria should remain oxidized both in the presence and absence of cyanide. This did occur in the tissues that they tested, but the experiment is not too convincing given the multiplicity of flavoproteins in plant mitochondria and the general lack of knowledge about them. Perhaps the best evidence indicating a branched chain rather than two separate ones is the fact that in the presence of ATP and cyanide, reversed electron transport seems to take place from cytochrome c to the alternate pathway (16).

b. The Branch Point

The nature of the branch point has long been a preoccupation of researchers concerned with cyanide resistant respiration. For a

time it was thought that a flavoprotein was the most likely candidate. However it now appears that ubiquinone fulfills this role.

It is clear that the branch point must be located between phosphorylation sites 1 and 2 of the electron transport chain. Storey and Bahr (97) found that shunk cabbage (Symplocarpus foetidus) mitochondria had a ADP:O ratio of 0 when oxidizing succinate in the presence of cyanide, while under the same conditions a value of 0.7 was obtained when malate was the substrate. Other workers have confirmed these results (45). As well, since cyanide resistant respiration is also antimycin resistant respiration, the branch point must be before the b cytochromes.

The most direct approach in determining the branch point is the administration of an oxygen pulse to anaerobic, cyanide-inhibited mitochondria. The components that then become oxidized belong to the alternate path and the time course of their oxidations indicate their sequence. Unfortunately when this is done the reduced cyanide . . . a_3 complex turns over too rapidly to allow measurements of a type required to be made (93).

Storey (98) has recently repeated these experiments using carbon monoxide as an inhibitor of the cytochrome pathway. With shunk cabbage mitochondria he found that the ubiquinone and an unidentified flavoprotein became oxidized rapidly upon the addition of the oxygen pulse. The oxidation of these two components could be prevented by m-CLAM, an inhibitor of the alternate pathway. Therefore Storey concluded that ubiquinone was the branch point and that the unidentified flavoprotein was the first member of the alternate pathway.

There is however considerable interference from protein absorption when spectrophotometric measurements are done in the region where ubiquinone has its absorption peak, namely the ultraviolet region. It is therefore desirable to have other methods of confirming Storey's results.

Huq and Palmer (48) have used the pentane extraction method originally developed by Szardzowski (99) to extract ubiquinone from Arum mataculatum mitochondria. They found that removal of the ubiquinone caused a loss of both cyanide sensitive and insensitive respiration, which could be restored by duroquinone, or by reconstitution with ubiquinone 50. This would indicate the involvement of ubiquinone in cyanide resistant respiration, but does not prove it is the branch point. However a partial removal of the ubiquinone resulted in a loss of the cyanide resistant fraction without a loss in the cyanide sensitive fraction. This would suggest that there are separate pools of ubiquinone in the mitochondria. Bonner and Rich (18) have also expressed this opinion.

Recently Bonner and Rich (18) claim to have solubilized with deoxycholate, a quinone:O_2 reductase from skunk cabbage mitochondria. This reductase was inhibited by m-CLAM. If the enzyme isolation can be verified in other laboratories, it would no doubt clear up the questions surrounding the branch point and the role of ubiquinone in the alternate pathway.

c. Control of the Alternate Pathway

Since the alternate pathway and the cytochrome pathway coexist and since the alternate pathway is nonphosphorylating, it would seem that some mechanism for apportioning electrons between the two

pathways must exist. This problem has been the most thoroughly investigated by Bahr and Bonner (2,3).

Bahr and Bonner used a seemingly specific inhibitor of the alternate pathway, m-CLAM, in their investigation. The action of m-CLAM and other substituted benhydroxamic acids had been discovered earlier by Schonbaum, Bonner, Storey and Bahr (90). Bahr and Bonner plotted rate versus inhibitor concentration in the presence and absence of cyanide. From this data could be obtained p, a fraction indicating what portion of the alternate pathway was actually operating under the given experimental conditions. Under state 3 conditions in mung bean (Phaseolus aureus) mitochondria, the alternate pathway was not operating at all, while under state 4 conditions it was maximally active. With shunk cabbage mitochondria under the same assay conditions the values were 0.55 and 0.65 respectively. The primary controlling factor in the operation of the alternate pathway appeared to be the flux through the cytochrome pathway. Thus under state 4 conditions when activity of the cytochrome pathway was inhibited by a lack of ADP, electrons were shunted to the alternate pathway.

A mechanism by which control of this type could be achieved was also postulated by Bahr and Bonner (2,3). It is shown in Figure 2. It consists of two carriers at the branch point being in equilibrium with each other. The E'_o values of the carriers need to be such that under conditions were A, the carrier leading to the alternate pathway is fully or partially oxidized, B, the carrier leading to the cytochrome pathway is fully reduced.

Despite some difficulties with this mechanism, namely the fact that different Krebs cycle substrates occasionally have higher or lower

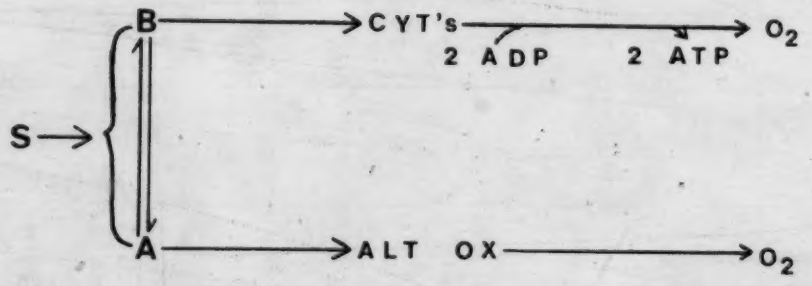


FIGURE 2. The branch point as envisioned by Bahr and Bonner.

Bahr and Bonner (3) envision the branch point to be composed of two separate electron carriers in equilibrium with each other. Further details are given in the text.

rates of oxidation by the alternate pathway than expected (45), no other general theories of control have been offered. One of the reasons for this is that no other methods for simultaneously measuring the activity of the alternate and cytochrome pathways have been found. Several authors have suggested that this is an experimental priority (72, 94).

It should be pointed out that one of the nice features of the mechanism discussed above is that it allows for the indirect control of the alternate pathway by the energy charge of the mitochondria. The phosphorylating cytochrome pathway is preferred to the alternate non-phosphorylating pathway when ADP is present (state 3) and the alternate pathway is preferred under high energy charge conditions (state 4).

d. Nature of the Alternate Oxidase

Complete knowledge of the nature of the alternate oxidase will probably not come until the enzyme has been isolated and purified. In this respect the recent solubilization reported by Bonner and Rich (18) is of considerable importance. They, however, have not as yet been able to characterize the proteins that they have solubilized.

Iron-sulfur proteins have long been implicated as possible components of the alternate pathway. Cheveau, Dizengemel and Lance (23) have studied the thermal denaturation of the electon transport chain in plant mitochondria. They have found that the cyanide-resistant pathway is consistently more sensitive to thermal denaturation than is the cytochrome pathway. They have also found that H_2S is released as the alternate pathway activity is lost. About 20% of the acid labile sulfur of the mitochondria of Arum Mataculatum was lost during the denaturation of the cyanide-resistant pathway.

Henry, Bonner and Nyns (44) have studied cyanide-resistant respiration in the yeast Saccharomycopsis lipolytica. They found that the cyanide-resistant respiration developed during the stationary phase of growth when the culture was aerated. The addition of Fe (III) to the culture medium increased the rate of appearance and the extent of development of the cyanide resistant respiration. Of the metals tested, only Fe (III) had this effect.

The above experiments together with those on the inhibition of the alternate pathway, to be discussed below, constitute the main body of evidence favouring the involvement of iron-sulfur proteins in the cyanide-resistant pathway. A good deal of effort has gone into investigations confirming suspicions by epr spectroscopy of the involvement of iron-sulfur proteins (74, 83, 86, 90). However, for the most part these experiments have proved unfruitful. Some authors (74, 90) have attributed part of the complex signal at g = 2.01 to the alternate oxidase. However, Rich, Moore, Ingeldew, and Bonner (86) have carefully examined this hypothesis and have discounted it, since there seemed to be no correlation between this signal and alternate pathway activity in the mitochondria from several plant species. Also, m-CLAM has no effect on this signal except at concentrations higher than that required for inhibition of the alternate pathway.

Henry (43) has found a signal at g = 4.03 in mitochondria from S. lipolytica, which he attributes to the alternate oxidase. Rich and Bonner have, however, been unable to find any evidence to link the signal at g = 4.03 to the alternate oxidase in higher plant mitochondria (83).

e. Inhibition of the Alternate Pathway

Inhibitors can provide a great deal of information about biochemical pathways and the nature of the enzymes involved. It has been known from some time now that various hydroxamic acid derivatives inhibit the alternate pathway (90). The most potent of these derivatives is m-iodobenzhydroxamic acid (K_i = 0.02 mM). However others including m-chlorobenzhydroxamic acid (K_i = 0.03 mM) and salicylhydroxamic acid (K_i = 0.06 mM) are also good inhibitors. The substituted benzhydroxamic acids are generally more inhibitory, perhaps because of their nonpolar nature. The inhibition is reversible in that it can be removed by washing.

It is natural to assume that the hydroxamic acids act by chelating a transition metal. All of them will complex with iron, particularly iron in the ferric state. However, this idea as been strongly challenged by Rich, Wiegand, Blum, Moore, and Bonner (87).

Rich et al (87) studied the effect of SHAM on several redox enzymes. Potent inhibitions (greater than 90%) of tyrosinase, L-α-glycerophosphate and horseradish peroxidase was obtained with 1 mM SHAM, a concentration often used to inhibit cyanide-resistant respiration. The inhibition was not related to the nature of the metal at the active site and could not be mimicked by other metal chelators. Kinetic analysis showed the inhibition of tyrosinase to be competitve with L-tyrosine. The authors concluded that inhibition of these enzymes was by competition with the phenolic substrate for the binding side on the protein. By analogy they feel that the alternate mitochondrial oxidase is probably inhibited by competition for its substrate, reduced ubiquinone.

An interesting point that arises as a result of this work is the specificity of SHAM in unpurified or whole cell systems. m-CLAM and SHAM have often been used in experiments where tissue slice respiration has been measured. Rich et al (87) point out that the tyrosinase enzyme in a potato slice has at least 100 times the oxygen-consuming capacity of the mitochondrial enzymes and is inhibited by SHAM at one order of magnitude less than the cyanide-resistant respiratory pathway is. Clearly the interpretation of such experiments is very difficult.

Other inhibitors of the cyanide-resistant pathway have also been found. Disulfiram (tetraetylthiuram disulfide) will inhibit the alternate pathway specifically and at a site different from the site inhibited by SHAM (36). Preliminary evidence implicates the formation of a mixed disulfide with a protein sulfhydryl group as the mode of action (36).

The quinone analog, dibromothymoquinone (2,5-dibromo-6-methyl-3-isopropyl-p-benzoquinone), inhibits both the cyanide sensitive and insensitive pathways in plant mitochondria (91). The inhibition pattern is complex and is not consistent with a site-specific mode of action. Part of its action may result from a general decrease in membrane fluidity (91). Nonetheless, inhibition by dibromothymoquinone has been cited as evidence for the involvement of ubiquinone in the alternative pathway (48).

f. Product of the Alternate Oxidase

There is some evidence to indicate that the end-product of the cyanide resistant pathway is H_2O_2 rather than H_2O. Rich, Boveris and Bonner (84) found that sub-mitochondrial particles inhibited by antimycin had rates of H_2O_2 production which correlated well with alternate

pathway activity. Whole mitochondria on the other hand had low rates of H_2O_2 production. This was attributed to the presence of endogenous catalase. Catalase has typically been regarded as a mitochondrial contaminant. However since even gradient purified mitochondria have been shown to possess some catalase (40, 84), it may have an intra-mitochondrial role. Although H_2O_2 production by whole mitochondria was low in Rich et al's (84) experiments, it was significantly higher when the alternate pathway was operating i.e. under state 4 conditions.

Production of H_2O_2 by the alternate oxidase has been used to explain the increase in respiration caused by cyanide in Chlorella protheocoides first discovered by Emmerson (31) and later confirmed by Grant and Hommersand (35). A simulation model (24) has shown that the simultaneous inhibition of cytochrome oxidase and catalase would increase O_2 consumption because of the stoichiometry involved in the production of H_2O_2. Since antimycin does not stimulate oxygen consumption in this organism, presumably because it does not inhibit catalase, the explanation seems reasonable.

g. Induction of the Alternate Pathway

In both plants and micro-organisms there are conditions that can increase the amount of cyanide-resistant respiration. The conditions which elicit this response vary from organism to organism.

In micro-organisms conditions that inhibit the cytochrome pathway, or inhibit its development, will induce the alternate pathway. Chloramphenicol for example, when included in the culture medium of Neurospora, will cause development of the alternate pathway (30). Chloramphenicol inhibits protein synthesis on mitochondrial ribosomes

and therefore inhibits the formation of some subunits of the b and a cytochromes. The inclusion of low concentrations of cyanide or antimycin A in the culture medium will also lead to the development of cyanide-resistant respiration in other organisms (63). With the yeast Candida utilis, a copper-deficient growth medium has been shown to stimulate the development of cyanide-resistant respiration (29). Presumably again this is because cytochrome oxidase cannot be synthesized because of the lack of copper, an essential component of the enzyme.

Another factor known to induce the alternate pathway in microorganisms is acetate. Troostenberg, Ledrut-Damanet and Nyns (101) showed that concentrated cell suspensions of Saccharomycopsis lipolytica excreted large amounts of acetate into the induction medium as they became insensitive to cyanide. Dilute cell solutions did not secrete acetate and did not become cyanide insensitive unless acetate was added to the medium. They hypothesized that a high rate of substrate oxidation coupled with a low rate of respiration resulted in the accumulation of an intermediary metabolite, acetate, which then triggered the induction process.

There seems to be only one reported instance where chloramphenicol has induced the alternate pathway in higher plants. The respiration of coleoptiles from rice seedlings that had been germinated in an aerated water solution containing 0.25 mg chloramphenicol/ml was about 90% resistant to cyanide (70).

Kano and Kumazawa (54) felt that pretreatment with low concentrations of HCN (10^{-14} M) for 2 to 12 hours leds to a subsequent increase in cyanide resistant respiration in wheat seedlings. They were unable

to show this with isolated mitochondria however (55) and it is difficult to ascertain from their experiments whether or not HCN in the environment actually led to an induction of the cyanide resistant pathway.

A copper deficient growth medium in a plant cell culture of Acer pseudoplatanus did not induce the alternate pathway (14). The K_i for cyanide declined and the amount of cytochrome oxidase as measured spectrophotometrically declined, but respiratory function was not impaired. Presumably this is because plant mitochondria normally have a large excess of cytochrome oxidase relative to other respiratory components.

The most familiar example of induction of the alternate pathway is the case of washed and aged potato slices (26, 38). Normally the respiration of mitochondria from the tubers of the white potato (Solanum tuberosum) is completely inhibited by cyanide. However if tissue slices are taken from the potato and maintained in an aerobic incubated medium for 24 hours, it is found that the mitochondria from these slices possess a cyanide-resistant, SHAM-sensitive respiration that can account for up to 40% of the mitochondrial respiration rate.

The reasons behind this phenomenon have never been fully elucidated, but the possibility exists that it may be as a result of the production of "wound" ethylene by the potato slices.

Cyanide resistance has also been produced by high levels of CO_2 in the ambient atmosphere and by ethylene. McCaig and Hill (71) reported that wheat coeloptiles grown in an atmosphere containing 25% CO_2 had significantly higher rates of cyanide resistant respiration than controls grown in a normal atmosphere. The effect was enhanced when the wheat seedlings were grown in an atmosphere containing only oxygen in addition to the carbon dioxide.

h. Cyanide-Resistant Respiration and Ethylene

The olefenic gas ethylene has long been known to have varied effects on plant growth (20, 65). It is now considered to be an endogenous plant growth regulator. Of special interest to many physiologists has been its effect on the respiration and ripening of fruit. Ripening fruit undergo a respiratory upsurge known as the climacteric (13). Associated with the climacteric is an increase in ethylene production. The climacteric and ripening can also be brought about artifically through an exogenous application of ethylene. The mechanism and cause of the climacteric are unknown.

Experiments by Lips and Biale (66) showed that the respiration of slices taken from climacteric avacadoes was not inhibited by cyanide or by amytal. The respiration of pre-climacteric slices was in fact stimulated by cyanide. As a result of their experiments they proposed a dual pathway concept for the oxidation of NADH in plant mitochondria. They soon abandoned this theory when they found that the mitochondria isolated from both climacteric and pre-climacteric slices were very sensitive to cyanide and amytal (11). A close examination of these results reveals that the mitochondria from climacteric slices were in fact slightly less inhibited than those from the pre-climacteric slices, but the experiments are too incomplete to form definite conclusions.

Solomos and Laties were the first to propose a link between the climacteric and cyanide-resistant respiration (93, 94). They found that in a large number of storage tissues, roots and fruits of various plants, both ethylene and cyanide, stimulated respiration. They felt that the ethylene exerted its action by a direct activation of the cyanide resistant respiratory pathway. However they were unable to

explain why isolated mitochondria from potatoes failed to show any indication of a cyanide resistant pathway.

It now seems that another explanation is possible. Rychter, Janes, and Frenkel (89) have shown that if mitochondria are isolated from potato tubers after they have been treated with either ethylene or cyanide, a significant amount of cyanide resistant respiration is present. They feel that ethylene stimulates the production of proteins responsible for cyanide-resistant respiration. They suggest that ethylene can be considered an inducer of the alternate pathway. There are still some questions, however, about the involvement of cyanide-resistant respiration in the respiratory climacteric. They have arisen because of failure of m-CLAM to inhibit respiration in tissue slices during the climacteric (65, 100).

i. Physiological Significance of Cyanide-Resistant Respiration

Except in the case of the Arum family where cyanide resistance seems to be very definitely associated with heat production (85), no clear-cut physiological roles for the cyanide-resistant respiratory pathway have been found. There are however many possibilities.

One of the most straightforward is that the cyanide-resistant pathway serves as an alternate pathway when conditions are unfavorable for the cytochrome pathway. Inhibitors of the cytochrome pathway abound in nature. Hydrogen cyanide can be produced by over 750 species of plants, as well as by numerous micro-organisms and fungi (53). In addition, compounds such as antimycin, hydrogen sulfide and carbon monoxide can also occur in nature (64, 112). The importance and significance of an alternate respiratory pathway under these conditions becomes obvious. In support of this hypothesis an experiment by Rissler and

Millar (88) can be cited. They found that exposure of the fungus Stemphylium loti to cyanide led to an induction of the alternate pathway. This pathway then seemed to provide the energy for the induction of formamide hydrol-lyase activity, which then detoxified the cyanide. The inclusion of SHAM with the cyanide prevented the induction of this enzyme.

Another experiment in this regard was carried out by Passam (80). He found that mitochondria isolated from cyanogenic cassava tubers were quite resistant to cyanide. Moreover, the degree of cyanide-resistance was correlated with the cyanide producing potential of the cultivar in question.

Alves, Heisler, Kissing, Patterson and Kalan (1) have found that the production of sesquiterpenoid stress metabolites in potato tubers was enhanced by ethylene: oxygen mixtures, conditions which induce the alternate pathway. The effect was suppressed by SHAM. Such an experiment raises the possibility that the alternate pathway or products arising from the alternate pathway are involved in stress metabolite production. There are may peroxidase enzymes in plants and it is possible that some utilize H_2O_2 produced by the alternate pathway.

A final hypothesis concerning the role of the alternate pathway and one that is gaining more acceptance (77) is that cyanide-resistant respiration serves in some way to decontrol respiration. In general plant mitochondria are believed to have two functions: 1) to generate ATP, and 2) to generate organic acid intermediates for biosynthetic reactions. In plant cells the first function can be served equally well by chloroplasts and photophosphorylation. One then might expect that the second to have more importance. The presence of the

alternate pathway would allow Krebs cycle reactions to occur in the presence of a high energy charge. Present knowledge concerning the control of the alternate pathway indicates that electrons could be switched back to the phosphorylating pathway should the ADP level of the cell increase. The alternate pathway would be aided in this role by the non-phosphorylating NADH dehydrogenase that Palmer (77) claims also exists in plant mitochondria. Another factor important to this scheme is the intramitochondrial presence of malic enzyme. It allows the mitochondria to start with any Krebs cycle intermediate and convert it to any other Krebs cycle intermediate. A final requisite of this scheme is that the Krebs cycle enzymes cannot be inhibited by ATP. This may not be the case (92).

There is a limited amount of experimental evidence to support the above hypothesis. Von Willert and Schwobel (105) have found that when leaves of the succulent plant Mesembryantheum crystallium shift from a C_3 photosynthetic mechanism to CAM photosynthetic mechanism, the mitochondria undergo a number of changes. Mitochondria from the CAM leaves have lower RCR and ADP:O ratios and much greater insensitivity to cyanide than leaves with a C_3 photosynthetic mechanism. These authors believed that this change allowed the mitochondria to convert malate to pyruvate, a process essential to the CAM photosynthetic mechanism, without a correspondingly higher ATP production. Mitochondria have already been shown to be involved in malate metabolism in C_4 plants.

Mitochondria have also been shown to be involved in the conversion of succinate to oxaloacetate in the endosperm of fat containing seeds such as castor bean (6). Succinate is derived from the glyoxalate cycle and transported to the mitochondria. The oxaloacetate

is not further metabolized but is converted by way of phosphoenolpyruvate and reversed glycolysis to sucrose for export to the developing axis. Although this process does not necessarily require a non-phosphorylating oxidation pathway, it does illustrate plant mitochondria playing a role in intermediary metabolism. There may be other instances where such a role could require a non-phosphorylating pathway.

C. Germination Processes in Pea Seeds

The aim of this study was to study cyanide-resistant respiration in germinating pea seeds. A great deal of information and experience on the respiratory reactions in germinating pea seed had already been accumulated in the laboratory and it seemed desirable to expand this to include the cyanide-resistant pathway. Cyanide resistance had not been studied extensively in pea seeds prior to this. A number of questions were addressed including the extent of cyanide resistance in isolated mitochondria as germination progressed, the relationship of cyanide resistance to other oxidation pathways, the role of ubiquinone in cyanide-resistant respiration, and the possible effects of external environmental factors on the development of the cyanide resistant respiration.

Because of the nature of the study, a brief review is given on some processes occurring in germinating pea seeds.

The most extensive study done on the germination of pea seeds was perhaps one done by Bain and Mercer (4). They studied ultrastructural changes in the cotyledons by electron microscopy and related changes occurring at this level to other anatomical and physiological changes.

They were able to distinguish three phases in the germination process. Phase 1 extended from the onset of imbibition to just before the start of shoot elongation. At the start of this phase the respiration rate was low and mitochondrial structure was not readily discernible. However shortly after the completion of imbibition the respiration rate rose dramatically and the outer double mitochondrial membrane and inner cristae were readily distinguished. Shortly before the end of phase 1, the respiration rate fell slightly.

Phase 2 consisted of shoot development and emergence. Lateral root formation was also initiated at this time. Some transport of reserve material from the cotyledons to the young axis also took place. The respiration rate remained relatively constant. Towards the end of phase 2 the cristae of the mitochondria seemed to become disorganized.

Phase 3 was characterized by the greatest loss of reserve material from the cotyledons. It is believed that most of the reserve material is transferred as sucrose and as amides such as glutamine and asparagine. The respiration rate increased somewhat during this period. The internal structure of the mitochondria was obscure but the outer double membrane appeared to remain intact.

Bain and Mercer also studied the relationship between the cotyledons and the developing axis (5). They found that the young seedling was dependent on the cotyledons long after its morphological development would indicate otherwise: i.e. well into phase 3. They also found that the presence of the young axis was necessary during the first 48 hours of germination. If it was removed normal development of the mitochondria and other ultrastructural features did not take place. This had earlier been noted by Young, Huang, Vaneko, Marks and Varner (116).

Solomos, Malhotra, Prasad, Malhotra, and Spencer (95) carried out a similar study on mitochondrial development in germinating peas. They found that the respiratory activity of mitochondria isolated from cotyledons shortly after the onset of imbibition was limited. It increased rapidly after the first two days though, and higher rates of substrate oxidation as well as good RCR and ADP:O ratios were obtained after 4 to 6 days of development. Thereafter the values declined somewhat as the cotyledons senesced.

In their studies Solomos et al (95) found that many of the mitochondria isolated shortly after the onset of imbibition banded at a heavier density than expected on a sucrose density gradient. These heavy mitochondria disappeared as the cotyledons developed. They therefore hypothesized that new mitochondria were not synthesized in the germinating peas but rather these heavy mitochondria were pro-mitochondria which subsequently underwent development.

To test this hypothesis Malhotra, Solomos, and Spencer (69) used radioactive precursors and measured the incorporation of these into mitochondrial DNA and protein. They found that ^{14}C leucine was incorporated into mitochondrial protein, but ^{3}H thymidine was not incorporated into mitochondrial DNA. They concluded therefore that a net synthesis of new mitochondria did not take place. This idea has been supported by others (75).

II. MATERIALS AND METHODS

A. Plant Material

1. Routine Germination Conditions

Cotyledons from seedlings of the garden pea (Pisum sativum L. var. Homesteader) were used in this study. Large lots of seed (5 kg) were purchased from a wholesale seed distributor (Seed Center, Edmonton) and were kept under refrigeration until needed.

The seeds were soaked in tap water for 6 hr. They were then planted in horticultural grade vermiculite to a depth of 1 1/2 inches. Broken, damaged and diseased seeds were discarded. Germination was in a growth chamber in the dark at 27 $\pm$ 2°C. The peas were watered daily and a high relative humidity was maintained throughout the growth period.

After the appropriate germination period the peas were harvested. Again, diseased and poorly germinated plants discarded. The seed coats were removed and the cotyledons were separated from the epicotyl and hypocotyl. The cleaned cotyledons were washed with tap water, rinsed once or twice with deionized water and then stored on ice until the mitochondria were isolated.

2. Alteration of Germination Conditions

a. Inclusion of Chloramphenicol

An experiment was done whereby chloramphenicol was included in the growth medium. This was accomplished by soaking the seeds in an aerated solution containing 0.5 mg/ml chloramphenicol for 24 hours. The

seeds were then transferred to a tray and were grown on filter paper for four days further. The filter paper was kept moist with the chloramphenicol solution. Temperature and light conditions were as above.

b. Inclusion of Azide

Sodium azide was included in the growth medium in the same way as chloramphenicol. Two concentrations were used: 10^{-4} M and 10^{-6} M.

c. Treatment with Light

Conditions in this treatment were the same as the normal germination conditions except that continuous light was substituted for continuous darkness.

d. Treatment with Ethylene

The seedlings in this treatment were grown in large respiratory jars. About 7.5 grams of dry pea seeds were placed in a jar and covered with vermiculite. The vermiculite was thoroughly wetted with tap water and excess water was poured off. The jar was then sealed and purified ethylene was injected into the jar to give a final ethylene concentration of near 100 ppm. The cotyledons were harvested six days later.

An alternate system was also used in some experiments. Peas were planted into a large (33 liter) plexi-glass growth chamber sealed except for two ports. A stream of purified air containing 112 ppm ethylene was then passed over the seeds for six days. The flow rate into the chamber was approximately 100 ml/min.

B. Isolation of Mitochondria

1. Preparation of Washed Mitochondria

The isolation procedure followed here was basically that of Phillips (81). All operations were carried out between 0 and 4°C. Approximately 250 grams of peas were ground in cold mortar and pestle for 8 min. The following grind medium (300 ml) was used: 0.5 M mannitol, 5 mM EDTA, 0.5% BSA, 0.05% cysteine and 50 mM Tes. The pH was adjusted to 7.4 at 20°C with Tris. The brei was filtered through one layer of miracloth and the filtrate was then centrifuged at 700 g for 7 min. The supernatant layer was recentrifuged at 21,000 g for 5 min. The supernatant layer, including the fluffy white layer surrounding the dark brown mitochondrial pellet, was removed with a pasteur pipet connected to a vacuum line. The mitochondria were then resuspended in a wash medium consisting of 0.3 M mannitol, 0.3% BSA and 25 mM Tes brought to pH 7.2 at 20°C by the addition of Tris. The centrifugation steps were repeated, and the mitochondria were suspended in the following medium: 0.3 M mannitol, 4 mM $MgCl_2$, and 50 mM Tes with the pH adjusted to 7.2 at 20°C with Tris.

2. Preparation of Zonally Isolated Mitochondria

The zonal procedure of Hamman and Spencer (41) was followed with some modifications. The initial steps up to and including the 700 g centrifugation step were identical to the above procedure. The supernatant layer from the low speed centrifugation was then edge-loaded into a chilled Ti-XIV zonal rotor spinning at 1500 rpm in a Beckman Spinco L2-65B centrifuge. Following this a sucrose-step gradient was loaded

into the rotor. The gradient consisted of 100 ml each of 33%, 37.5%, and 43% (w/w) sucrose, and sufficient 48% (w/w) sucrose to fill the rotor. The gradient also contained 0.1% (w/w) BSA and 1% (w/w) Tes, with the pH adjusted to 7.4 at 20°C with Tris. Centrifugation was for 1 hour at 37,000 rpm. At the end of this time the rotor was center-unloaded by use of a 50% (w/w) sucrose solution. Protein peaks were monitored with a Pharmacia UV Duo Monitor (Model 200). The protein peak at the interface of the 37.5% and 43% steps was saved. This fraction was slowly diluted with a buffered solution (0.1% BSA, 25 mM Tes, with pH adjusted to 7.2 at 20°C with Tris). This suspension was centrifuged for 10 min. at 19,000 g. The mitochondria were then resuspended in a medium consisting of 0.3 M mannitol, 4 mM $MgCl_2$, and 25 mM Tes, with the pH adjusted to 7.2 at 20°C with Tris.

C. Measurements of Mitochondrial Integrity

1. Succinate-Cytochrome c Reductase

Succinate-cytochrome c reductase activity was measured by the method of Douce et al (27). Mitochondria were added to 3 ml of succinate assay medium (0.3 mannitol, 5 mM phosphate, 5 mM $MgCl_2$, 0.75 mg/ml BSA and 50 mM Tes, with the pH adjusted to 7.2 at 20°C by Tris). The assay medium also contained 0.02 umoles of cytochrome c and was 0.5 mM in KCN. The reaction was initiated by the addition of succinate to a final concentration of 8 mM and was followed by observing the increase in absorbance at 550 nm on a Cary 15 recording spectrophotometer.

Osmotically burst mitochondria were assayed in a 10 mM phosphate buffer, pH 7.2 at 20°C.

2. NADPH-Cytochrome c Reductase

This activity was measured in 3 ml of assay medium that was made 0.5 mM in KCN and contained 0.02 umoles cytochrome c and 0.02 umoles NADPH. The reaction was followed by recording the increase in absorbance at 550 nm.

D. Respiratory Measurements

Oxygen uptake was measured with a Yellow Springs Instrument Company Model 53 oxygen monitor connected to a Beckman 100 mv potentiometric recorder. The procedure and media were those of Solomos et al (95).

The standard assay medium consisted of the following: 0.3 M mannitol, 4 mM $MgCl_2$, 5 mM phosphate, 0.75 mg/ml BSA and 50 mM Tes, with the pH adjusted to 7.2 at 20°C by the addition of Tris. The respiratory substrate was included in the assay medium. This was usually either 8 mM succinate, 8 mM ∝-ketogluturate, 10 mM L-malate. The sodium salt of these acids was used. The malate and ∝-ketoglutarate media contained 0.1 mM thiamine pyrophosphate and the ∝-ketoglutarate medium contained 5 mM malonate as well.

In a typical assay, 3 ml of assay medium containing the appropriate substrate was added to the reaction vessel. This was allowed to equilibrate for 3 to 5 minutes. The temperature in the water bath surrounding the reaction vessel was maintained at 25°C. Following equilibration the oxygen probe was inserted into the reaction vessel and the recorder was set to read 100% saturation. Complete oxygen saturation of the assay medium was assured by keeping the assay medium cooled prior to adding it to the reaction vessel. Between 0.5 and 1.5 mg of mitochondrial protein

was then added, followed by 10 to 20 ul of 0.03 M ADP. From 2 to 4 cycles of state 3, state 4 conversions were initiated.

When inhibitors were used they were usually added after the second or third ADP addition. Potassium cyanide was routinely made up as 0.02 M solution in water. Thirty ul of this solution was added to the reaction vessel to give a final KCN concentration 0.2 mM.

SHAM was dissolved in dimethyformamide and 30 ul were added to make the final concentration in the reaction vessel 1 mM. Chloroquine was dissolved in water and handled similarily. Rotenone was dissolved in dimethylformamide. Antimycin A was dissolved in 95% ethanol and from 5 to 10 ul of solution was used to give a final concentration of 0.4 ug/ml.

Occasionally, at the completion of the run a small amount of dissolved sodium diothionite was added to the reaction vessel. This insured that the baseline on the recorder was indeed 0. It was assumed that the oxygen saturated assay medium contained 275 nmoles O_2/ml. The calibration procedure suggested by Estabrook (32) was used to check this value. In this experiment, the amount of oxygen consumed by frozen mitochondria oxidizing known amounts of NADH was measured. This method indicated that the value for the saturated solution was probably between 225 and 240 nmoles O_2/ml. However since the 275 figure had been used in previous calculations and in other studies in the laboratory, it was retained.

When duroquinol or menadiol were used as respiratory substrates, they were first prepared by reduction with sodium borohydride. A few grains of sodium borohydride were added to a 50 mM solution of the oxidized substrate, which was dissolved in 95% ethanol. A few drops of

HCl then added to insure reduction and to eliminate the remaining borohydride. From 10 to 20 ul of this solution were added to the reaction vessel. Menadiol and duroquinonol oxidations were carried out at pH 6.0.

E. Extraction of Coenzyme Q

The procedure for the extraction of ubiquinone from the mitochondria was basically that of Szarkowska (99). Washed mitochondria from 6 day cotyledons were resuspended in 0.15 M KCl at a concentration between 10 and 20 mg protein/ml. About 10 ml of this solution was quick frozen in a dry ice-acetone bath. The frozen suspension was then lyophilized for approximately 8 hours using a Virtis lyophilizer. After the lyophilization procedure was complete, the mitochondria were resuspended in pentane with a teflon coated pestle using a Sorvall Omnimixer and were shaken for five minutes in a glass-stoppered test tube. The mixture was then centrifuged at high speed on a small table centrifuge for 3 minutes. The pentane was removed and the procedure was repeated. The extraction procedure was repeated five times. The residue after extraction was dried under vacuum at room temperature for 30 minutes. All other operations were carried out between 0 and 4°C.

Reincorporation of the ubinquinone, either the previously extracted ubiquinone or commercially obtained Coenzyme Q_6 was accomplished by the reverse procedure. A small amount of ubiquinone containing pentane (2 - 3 ml) was shaken in a glass-stoppered test tube with the extracted mitochondria. The pentane was removed following centrifugation and the mitochondria were lightly washed with pentane. The mitochondria with reincorporated Coenzyme Q were then dried under vacuum at room temperature.

The lyophilized, extracted and reincorporated mitochondria were homogenized in the mitochondrial suspending medium and assay in the same manner as other mitochondria.

F. Measurements of the Redox State of Coenzyme Q

The procedure followed was basically that of Kroger and Klingenberg (59). Between 250 and 500 ul of a highly concentrated mitochondrial suspension (10 to 20 mg protein/ml) were added to 1 ml of mitochondrial assay medium. Succinate was used as the respiratory substrate. The mixture was incubated for 5 minutes at 25°C. The reaction was terminated by the rapid addition of 5 ml of 60% methanol: 40% petroleum ether (v/v) mixture. The resulting suspension was then rapdily mixed for 1 minute with a Vortex mixer. The petroleum ether was removed with a Pasteur pipet after a brief centrifugation (1 to 2 min) in a table centrifuge. The extraction procedure was repeated with 4 ml of petroleum ether. The combined petroleum ether extracts were then washed once with 95% methanol. The petroleum ether was removed by evaporation at reduced pressure. The remaining material was dissolved in a 4:1 ethanol: heptane mixture under nitrogen. The absorbance spectrum between 220 nm and 320 nm of this solution was taken on a Cary 14 recording spectrophotometer. A small amount of sodium borohydride was added and after a few minutes, another absorbance spectrum was taken. The oxidized minus reduced absorbance at 275 nm was determined.

G. Protein

The Lowry method (67) for determining protein was used. From 10 to 25 ul of the protein containing solution were added to 0.5 ml of 20%

TCA. This was then centrifuged at high speed in a small table centrifuge. The TCA was removed and the pellet was redissolved in 0.5 ml of 1 N NaOH and brought to a volume of 1 ml with distilled water. Five ml of a solution 0.01% in copper sulfate, 0.02% in sodium-potassium tartarate and 2% in sodium carbonate were then added. After 10 minutes 0.5 ml of Folin-phenol reagent was added. The absorbance was read at 750 nm 30 minutes later. A standard curve was prepared with human serum albumin.

H. Chemicals

All chemicals were of reagent grade and with the exception of the following were from Fisher Scientific Co. Cytochrome c (Type III), NADH, NADPH, ADP, Tes, duroquinone, menadione, chloroquine and cysteine were from Sigma Chemical Co., BSA, chloramphenicol and α-ketoglutarate, were from Calbiochem Corp. and salicylhydroxamic acid from Aldrich Chemical Co. Coenzyme Q_6 was obtained from Mann Research Laboratories. Purified gases were obtained from Matheson Co.

III. RESULTS

A. Morphological Development of the Peas

The growth of the peas was very rapid with the germination conditions used. Figure 3 illustrates this. The radicle emerged between 24 and 48 hours after the onset of imbibition. Thereafter the shoot system developed. The shoot emerged above ground between the third and fourth day of development. Because the growth chamber was kept dark, the shoot and leaf system did not develop fully. Lateral root formation began near day 5. The bent shape of the roots shown in the diagram occurred because of the nature of the growing container. Development after the fifth day consisted mainly of epicotyl elongation and root extension.

B. Mitochondrial Integrity and Purity

A comparison between zonally isolated and "washed" mitochondria is given in Table I. The former preparation was buff in color, while the latter was always distinctly brown. Zonally isolated mitochondria consistently had higher specific rates of oxygen consumption and had slightly better respiratory control ratios. However ADP:O ratios and the percentage of respiration inhibited by cyanide were similar in both preparations. The oxygen uptake of the zonally isolated mitochondria was completely inhibited by cyanide + SHAM, but a small amount of residual respiration remained in the case of the washed mitochondria.

The reduction of externally added cytochrome c by succinate is also shown in Table I. In mannitol-Tes buffer the reduction was less than 10% of the activity in phosphate buffer. This activity was somewhat

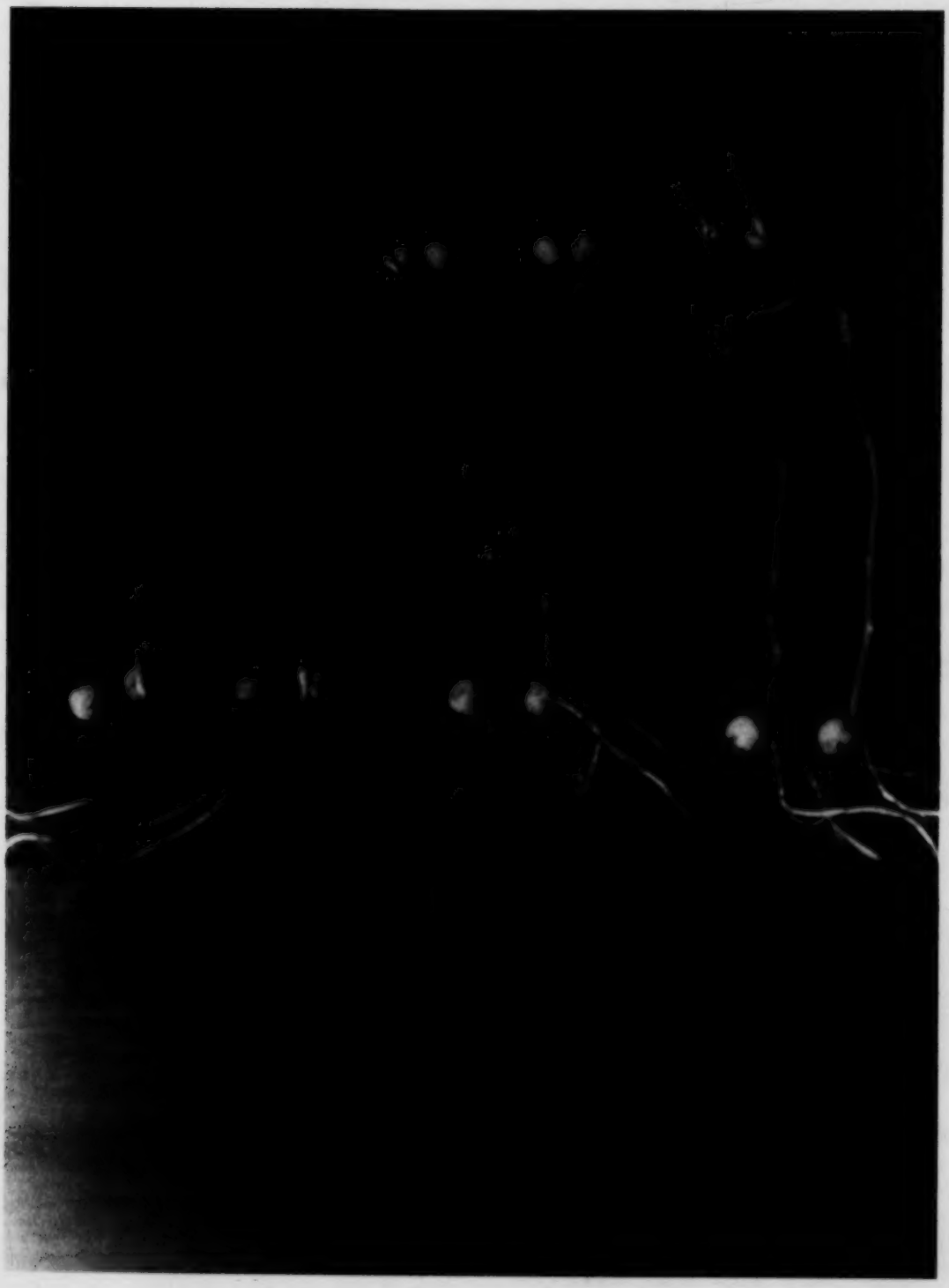


FIGURE 3. Morphological development of the pea seedlings.

Peas were soaked in tap water for 6 hours prior to planting. They were then planted in horticultural grade vermiculite and placed in growth chambers and kept at 27 ± 2°C in the dark for the appropriate period of time. The above illustration shows the pea seedlings at 1, 2, 3, 4, 5, 6, 8 and 10 days after the onset of imbibition (starting at the top left and proceeding from left to right).

TABLE I

A Comparison of the Respiratory Activities of "Washed" Mitochondria and Zonally Isolated Mitochondria.

Mitochondria were isolated as in the Materials and Methods section. A batch of 8 day old cotyledons was divided into two parts and carried through the respective procedures. Similar results were obtained when the experiment was repeated with 2 and 6 day old cotyledons.

Parameter	Washed Mitochondria	Zonally Isolated Mitochondria
Oyxgen uptake, state 3 (nmoles/min/mg protein)	117.7	181.7
Respiratory control ratio	2.42	3.38
ADP:O Ratio	1.37	1.38
Cyanide-sensitive respiration	88%	88%
Succinate-cytochrome c reductase in mannitol-TES buffer (nmoles cytochrome c reduced/ min/mg/protein)[a]	10	7
Succinate-cytochrome c reductase in 10 mM phosphate buffer (nmoles cytochrome c reduced/ min/mg protein)	154	173
NADPH-cytochrome c reductase (nmoles cytochrome c reduced/ min/mg protein)	72	32
Antimycin A-resistant NADPH-cytochrome c reductase (nmoles cytochrome c reduced/ min/mg protein)	20	11

[a]The millimolar absorbance coefficient used was 21.0 mM^{-1} cm^{-1} (6).

lower for the zonally isolated mitochondria than for the washed mitochondria.

C. Mitochondrial Oxidations

1. Oxidation of Succinate

The oxidation of succinate by zonally prepared mitochondria from 4 day old cotyledons is shown in Figure 4. Mitochondria prepared from cotyledons of this age typically had high rates of substrate oxidation with good RCR and ADP:O ratios. The oxidation of succinate was more rapid than that of other Krebs cycle intermediates. Oxidation rates were around 250 nmoles O_2 min.mg protein in state 3 and near 90 nmoles O_2/min.mg protein in state 4. The RCR was usually between 2 and 3 and the ADP:O ratio near 1.5. The respiratory control ratio was lower with succinate than with either -ketoglutarate or malate.

a. Effect of Cyanide

The effect of cyanide on succinate oxidation is also shown in Figure 4. The effect of cyanide was the same whether it was added during state 3, state 4, or to the uncoupled state. Uncoupling was achieved with 1.8 uM CCP. ADP added after the cyanide had no effect on the oxidation rate (not shown).

The concentration of cyanide required for inhibition was also investigated. The results are shown in Figure 5. Half-maximal inhibition was obtained between 4 and 10 uM KCN. However complete inhibition was not achieved even with 5 mM KCN.

b. Effect of SHAM

The effect of SHAM on the cyanide resistant oxidation is also

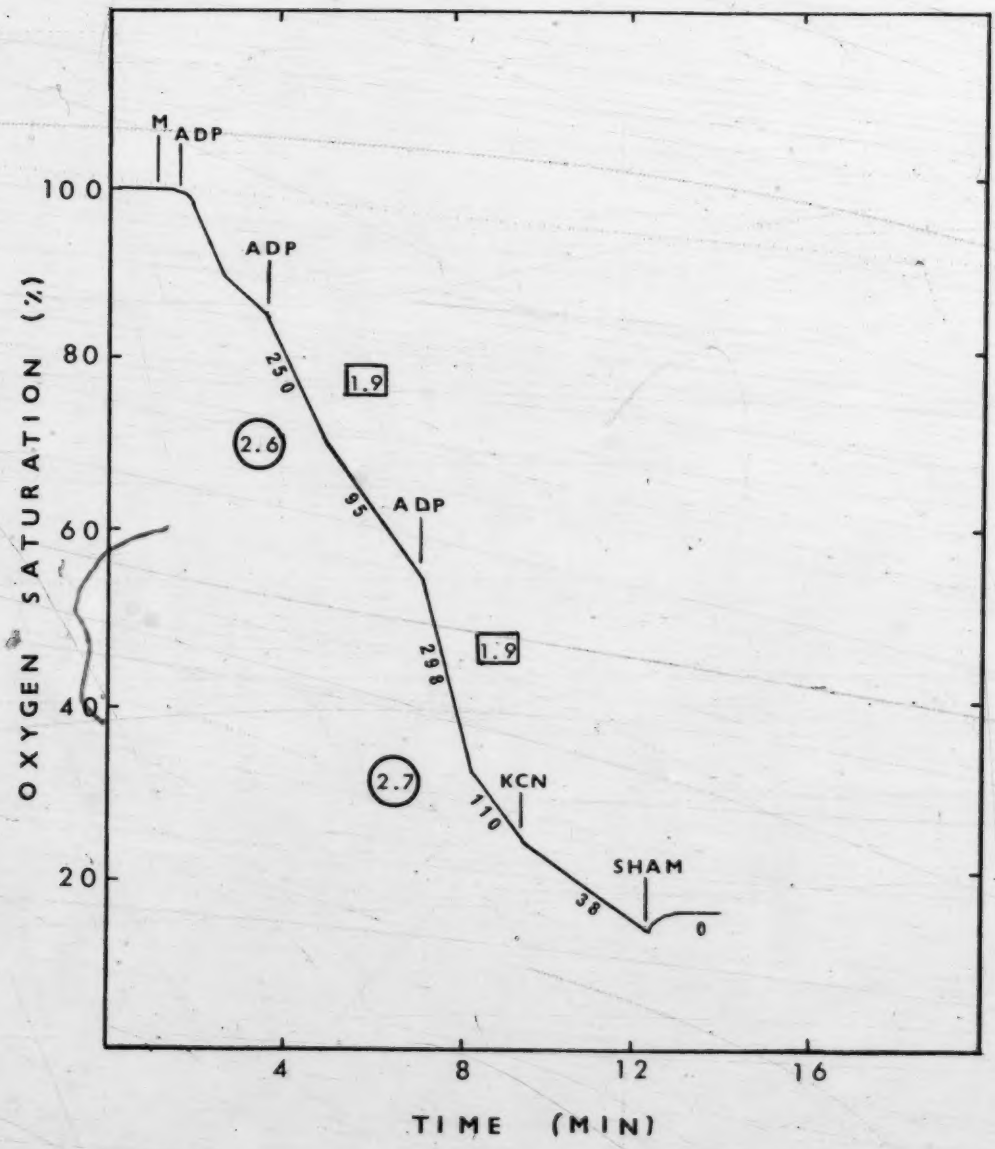

M
ADP
ADP
ADP
KCN
SHAM
250
95
298
110
38
0
1.9
1.9
2.6
2.7
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 4. Oxidation of succinate by pea cotyledon mitochondria.

Mitochondria from 4 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and numbers in rectangles to the ADP:O ratio.

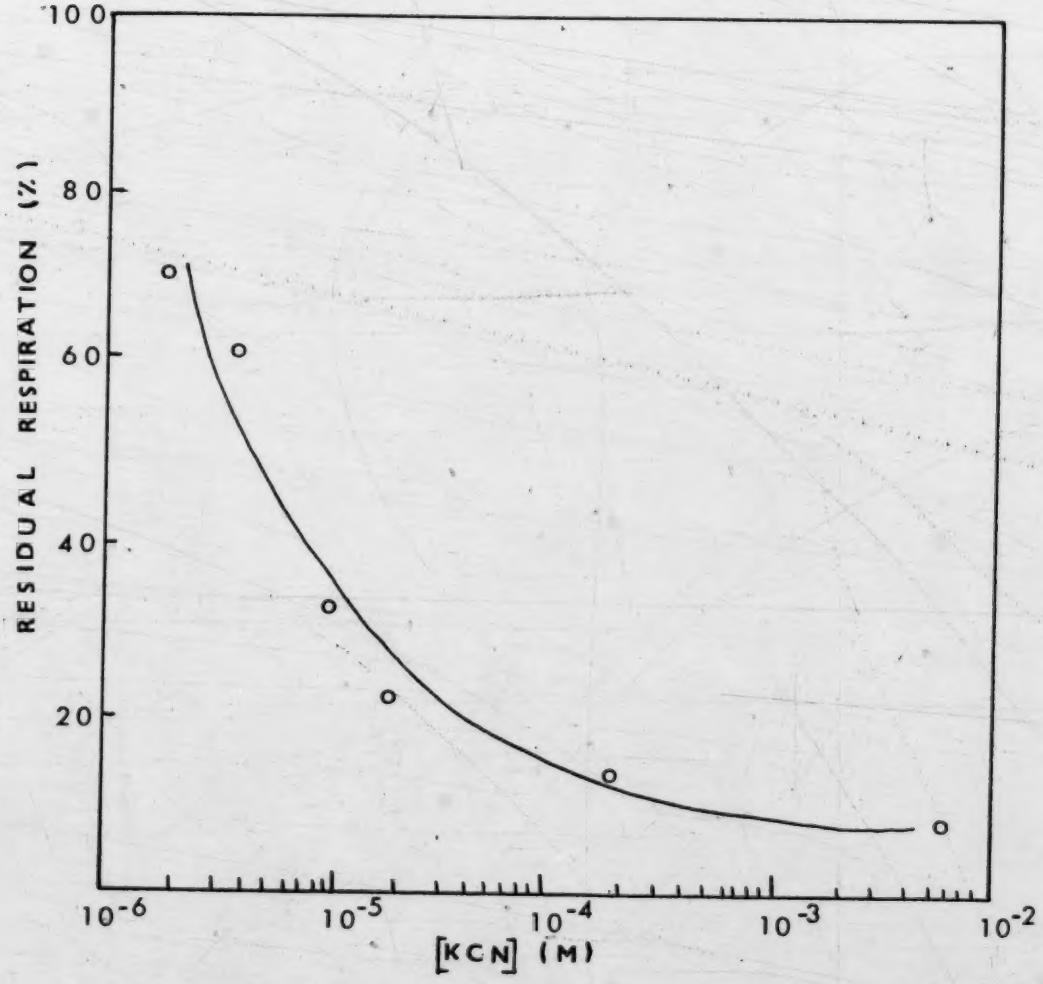

100
80
60
40
20
RESIDUAL RESPIRATION (%)
10-6
10-5
10-4
10-3
10-2
[KCN] (M)

FIGURE 5. The effect of cyanide on the oxygen uptake of pea cotyledon mitochondria.

Mitochondria from 6 day old cotyledons were prepared by zonal centrifugation and assayed polarographically. (See Materials and Methods). The respiratory substrate was 8 mM succinate. The experiment was repeated with another preparation from 6 day old cotyledons, and with preparations from 2 and 8 day old cotyledons.

shown in Figure 4. The cyanide resistant respiration was completely inhibited by SHAM in the case of zonally isolated mitochondria. A small amount of residual respiration (1 to 2%) occasionally remained with the washed preparation. In the absence of cyanide, the addition of SHAM to state 3 had little effect on the respiration rate (Figure 6) while addition of SHAM to state 4 caused a considerable decrease in the respiration rate. Complete inhibition with KCN and SHAM together was obtained regardless of the order of addition.

The inhibition versus concentration curve for SHAM is shown in Figure 7. Half maximal inhibition was obtained at approximately 0.17 mM SHAM. This curve was used together with the method of Bahr and Bonner (2,3) to determine the extent to which the cyanide-resistant pathway was actually operating during state 3 and state 4 respiration (Figure 8). The total respiration rate (V_T) was plotted against g(i), a function describing the activity of the alternate pathway at various inhibitor concentrations (Figure 8). The function g(i) was determined from the inhibition curve of the cyanide-resistant respiration by SHAM (Figure 7). The slope of the V_T versus g(i) curve yielded p, the fraction of the cyanide resistant pathway operating under the given conditions. p was determined to be 0.2 in state 3 and 0.5 in state 4. This fraction was approximately the same for 2, 4, 6, and 8 day old cotyledons.

2. Oxidation of α-Ketoglutarate

The oxidation of α-ketoglutarate as shown in Figure 9. The oxidation rate was considerably slower than that of succinate but higher RCR's were obtained. The ADP:O ratios were indicative of 4 coupling sites. Inhibition by cyanide lowered the ADP:O ratio by

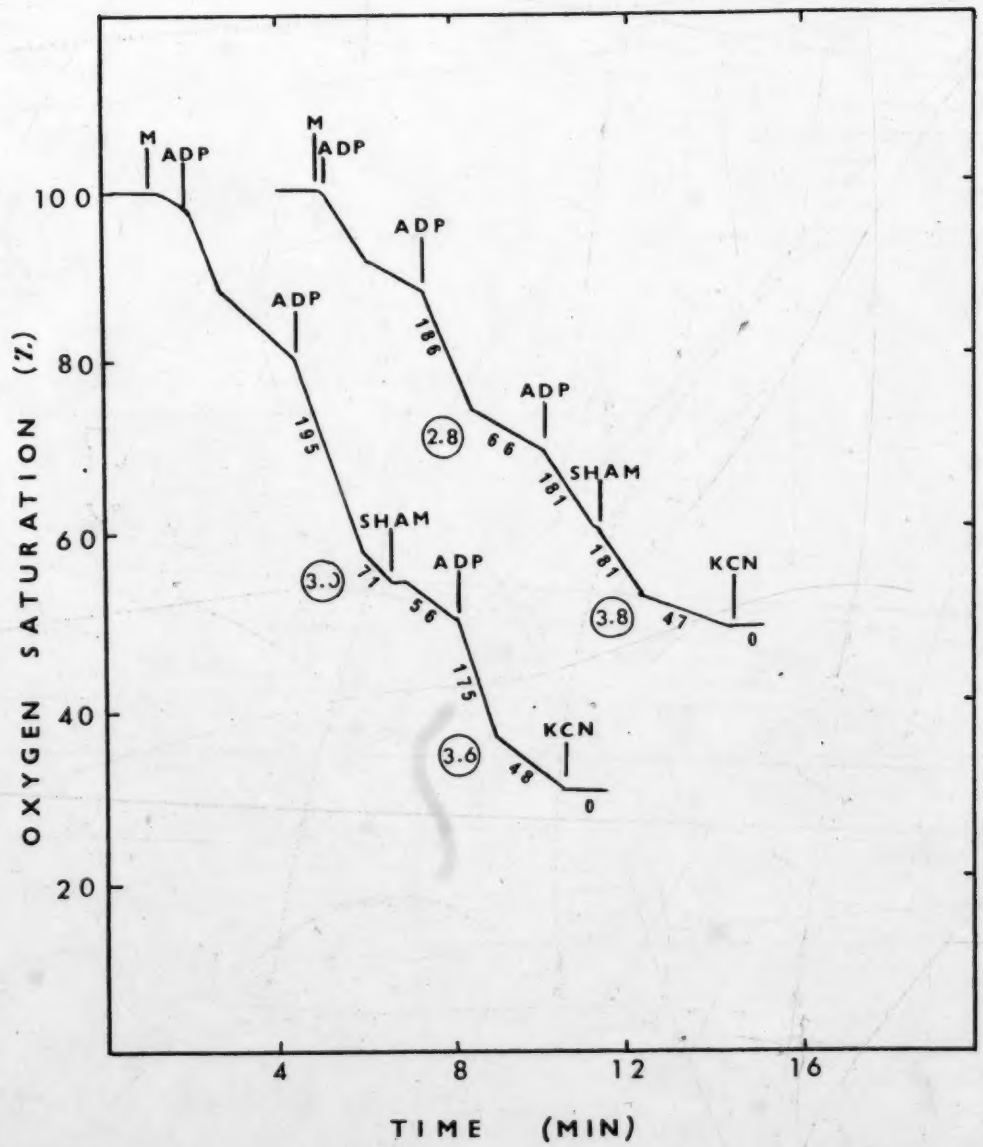

M
ADP
M
ADP
ADP
ADP
ADP
SHAM
SHAM
ADP
KCN
KCN
195
186
66
181
181
71
56
175
48
0
47
0
2.8
3.3
3.8
3.6
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 6. The effect of SHAM on state 3 and state 4 respiration.

Mitochondria from 6 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The final concentration of SHAM was 1 mM. The numbers alongside the traces refer to oxygen uptake in nmoles O_2 min . mg protein. The circled numbers refer to the RCR.

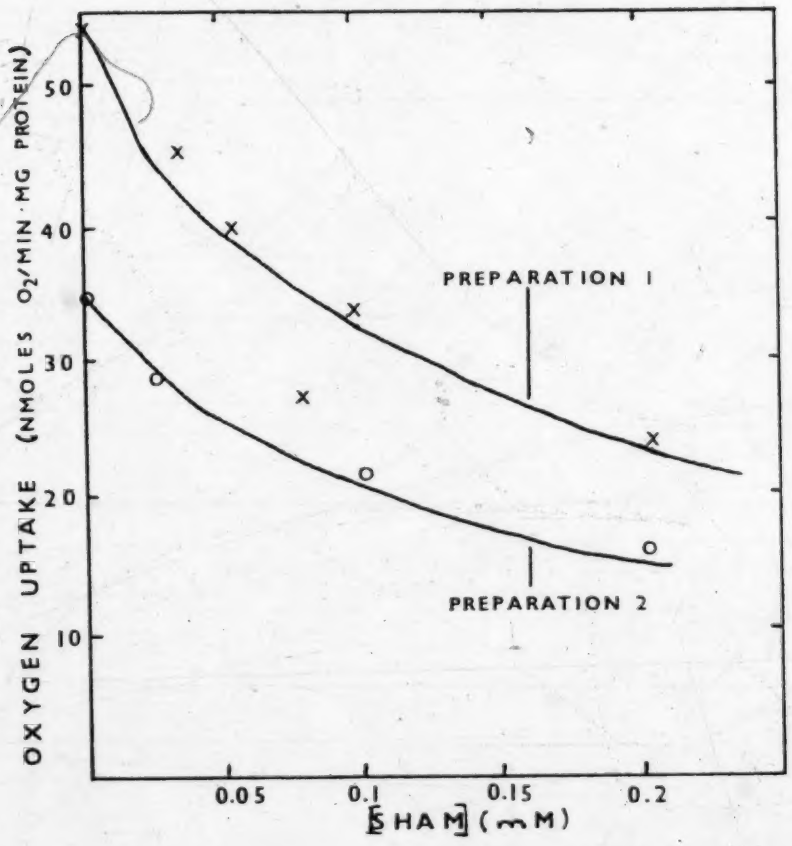


FIGURE 7. Effect of SHAM on cyanide-resistant respiration.

Washed mitochondria mitochondria were isolated from 4 day old pea cotyledons and were allowed to oxidize succinate. Varying concentrations of SHAM were added prior to the mitochondria. After the second period of state 3 respiration KCN was added to a final concentration of 0.5 mM.

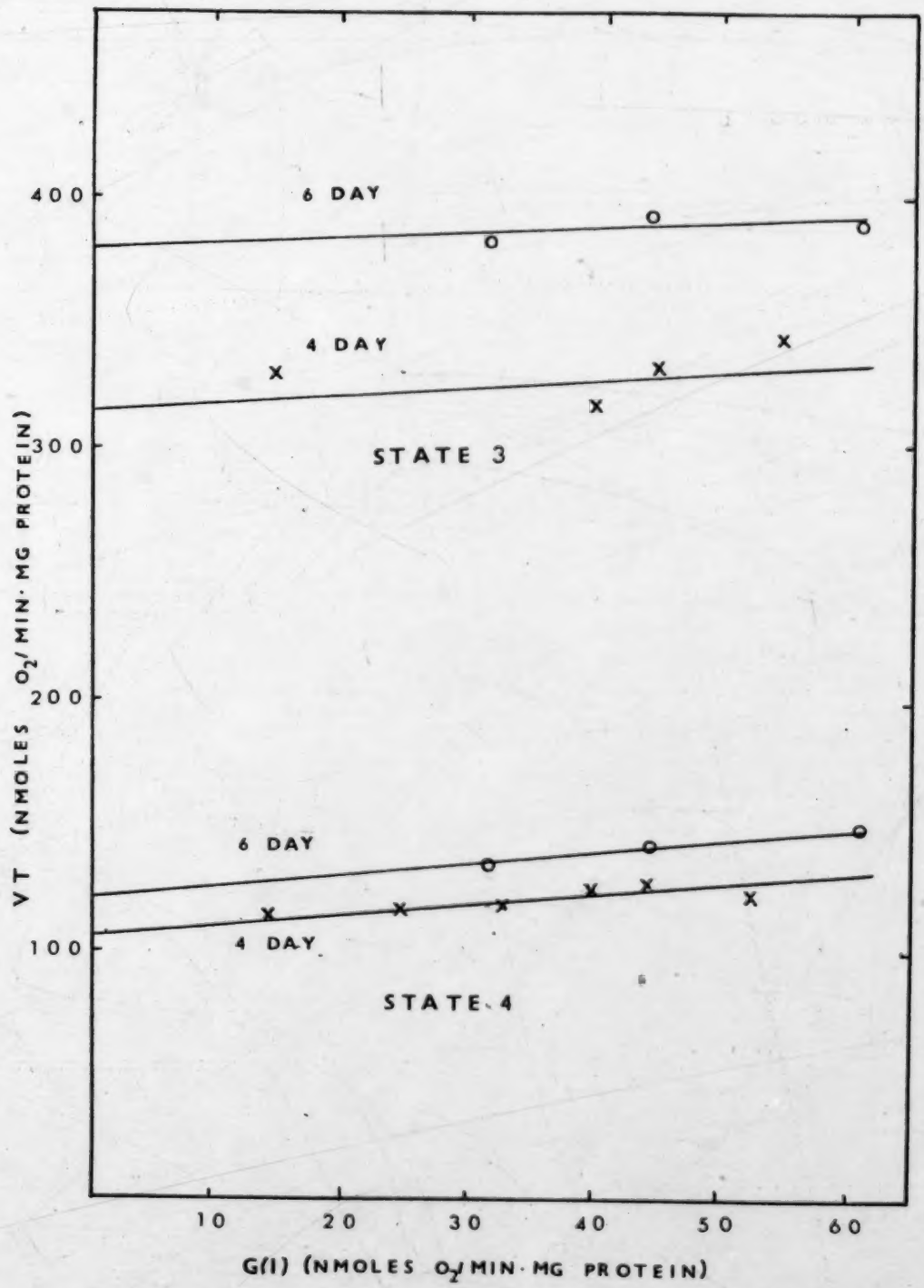

400
300
200
100
6 DAY
4 DAY
STATE 3
6 DAY
4 DAY
STATE 4
VT (NMOLES O2/MIN·MG PROTEIN)
10
20
30
40
50
60
G(1) (NMOLES O2/MIN·MG PROTEIN)

FIGURE 8. The respiratory rate in states 3 and 4 versus g(i).

Washed mitochondria were isolated from cotyledons of the appropiate age and were allowed to oxidize succinate. SHAM, at varying concentrations, was added prior to the mitochondria. The resulting oxidation rate was plotted against g(i), which for that particular concentration of SHAM, was determined from experiments like those shown in Figure 7.

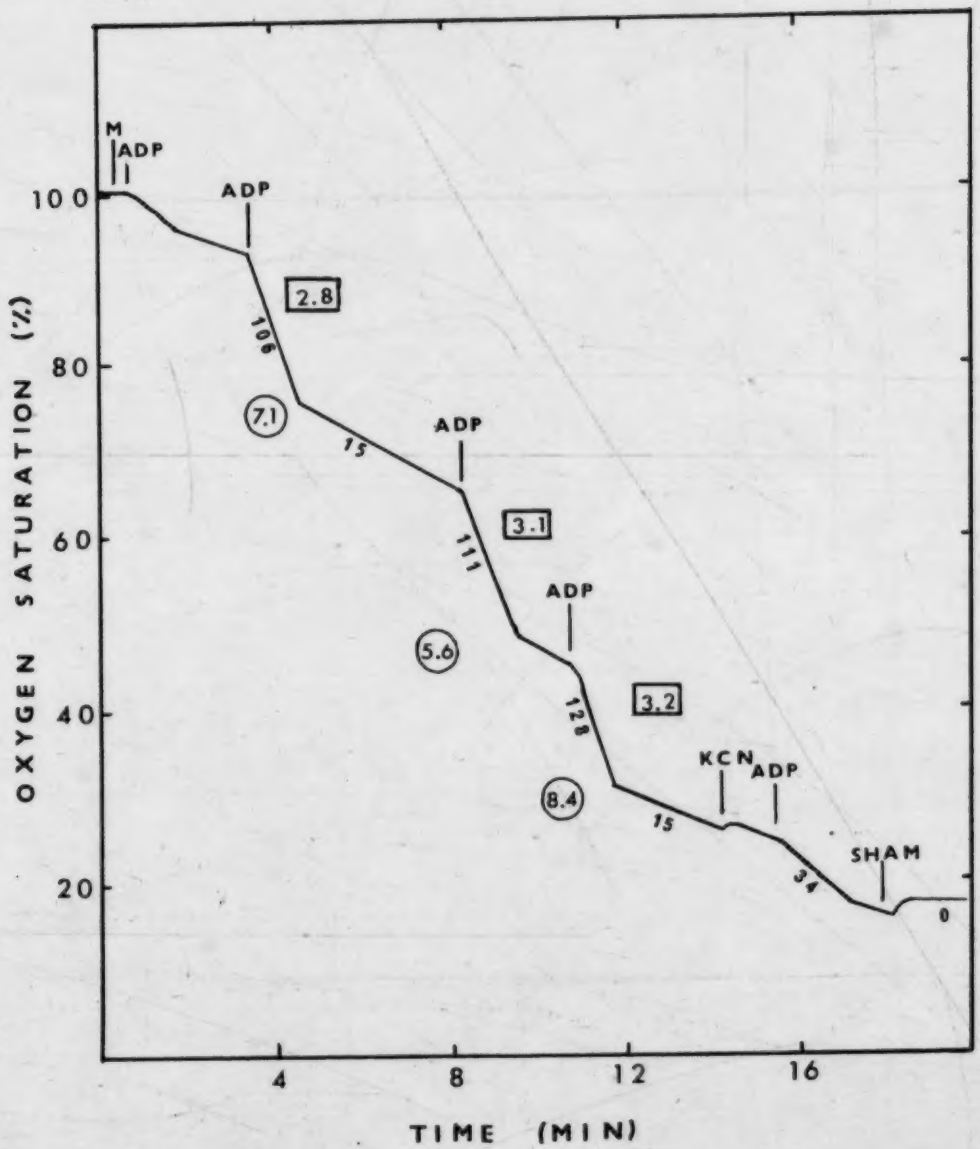

M
ADP
ADP
100
80
60
40
20
OXYGEN SATURATION (%)
2.8
108
7.1
15
ADP
3.1
111
5.6
ADP
3.2
128
8.4
KCN
ADP
15
SHAM
34
0
4
8
12
16
TIME (MIN)

FIGURE 9. Oxidation of α-ketoglutarate by pea cotyledon mitochondria.

Mitochondria from 4 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The numbers alongside the traces refer to oxygen uptake in nmoles O_2 min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

approximately 2 units. SHAM and cyanide together lowered the respiration rate to 0.

The possibility existed that the α-ketoglutarate and the cyanide might be reacting to form a cyanohydrin. This would mean that the effective concentration of the cyanide in solution would be lower, thereby accounting for part of the cyanide resistance. This possibility seemed unlikely since the cyanide-resistant respiration was inhibited by SHAM. A further experiment seemed to confirm this. Sodium arsenite was added to the mitochondria oxidizing α-ketoglutarate. All respiration was inhibited. Ascorbate-TMPD, which donates electrons directly to the cytochrome oxidase region, was then added and respiration resumed. Following this SHAM was added and the ascorbate-TMPD respiration was inhibited almost entirely. While this does not mean that cyanohydrin formation was not occurring, it does mean that its importance was minimal.

3. Oxidation of Malate

The oxidation of malate (Figure 10) was somewhat variable. A few preparations oxidized malate much more slowly than other preparations. However in general the oxidation of 10 mM malate was about 135 nmoles O_2/min.mg protein. The ADP:O ratio was about 2.3 and was decreased to less than 1 by the addition of cyanide.

Occasionally the state 4 oxidation rate with malate showed two phases: an initial slow phase followed by an abrupt change into a faster rate. Another point concerning the oxidation of malate was that the second and third state 3 rates were lower than the first. The opposite occurred with succinate and α-ketoglutarate.

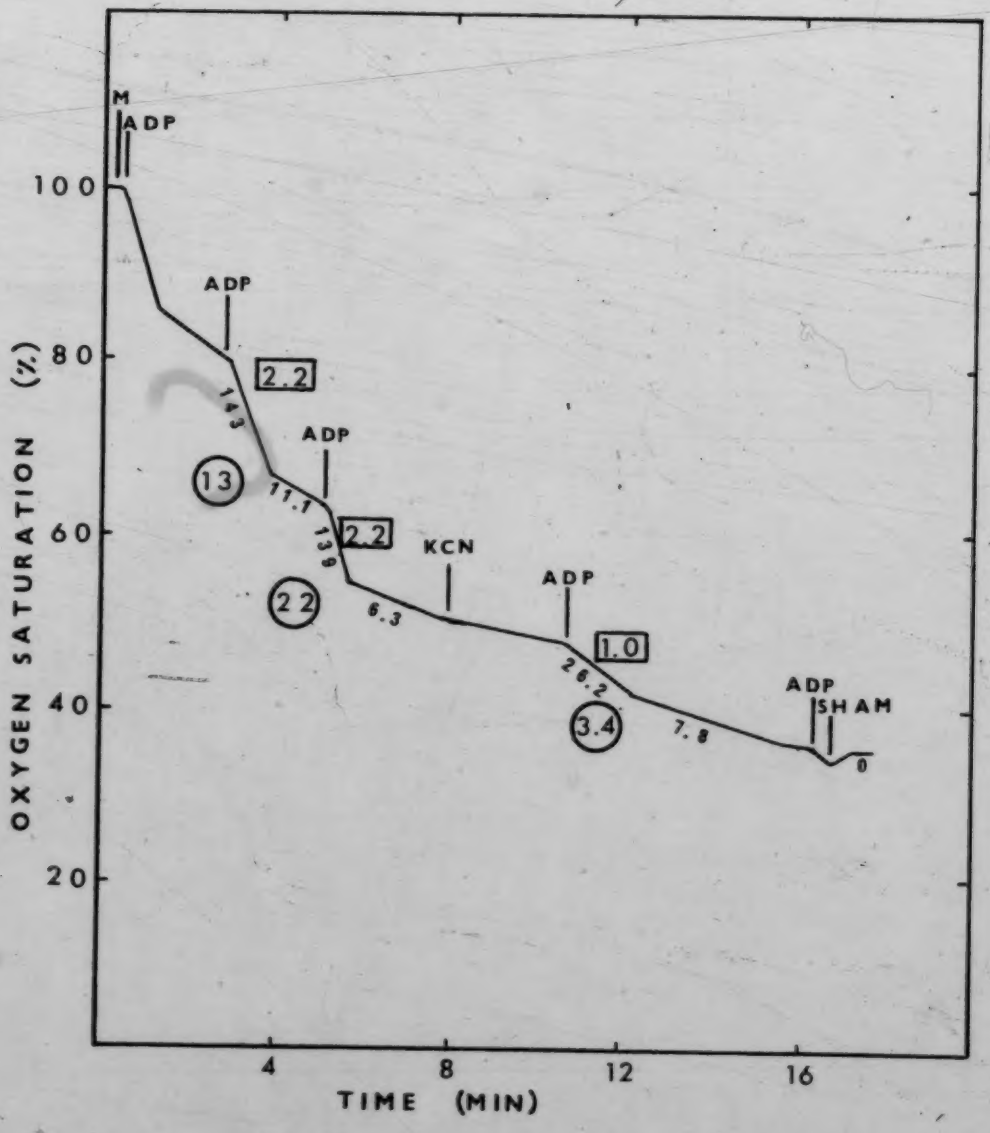

M
ADP
ADP
ADP
ADP
KCN
ADP
ADP
SHAM
2.2
2.2
1.0
14.3
11.1
13.9
6.3
26.2
7.8
0
13
22
3.4
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 10. Oxidation of malate by pea cotyledon mitochondria.

Mitochondria were isolated from 4 day old cotyledons by the zonal technique and assayed polarographically. (See Materials and Methods). The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

4. Oxidation of NADH

Externally added NADH was oxidized rapidly by the pea cotyledon mitochondria (Figure 11). The ADP:O ratio was near 1.5. A small fraction of the NADH oxidation was resistant to cyanide, and sensitive to inhibition by SHAM.

5. Oxidation of Citrate

The oxidation of citrate (Figure 12) proceeded very slowly but with good respiratory control. ADP:O ratios were near the theoretical 3. The cyanide resistant respiration rate was large when expressed as a percentage of the total respiration rate but on per mg protein basis, it was not greatly different from other respiratory substrates.

6. Oxidation of Ascorbate-TMPD

The oxidation of ascorbate-TMPD (Figure 13) proceeded extremely rapidly with poor respiratory control (RCR = 1.5). ADP:O ratios were less than one. Cyanide (0.2 mM) did not completely inhibit the oxidation of the ascorbate-TMPD mixture, but the remaining oxidation was only slightly inhibited by SHAM.

Addition of succinate after the cyanide caused a slight stimulation of the respiration rate. Most of this was sensitive to SHAM. An experiment was also conducted in which no ADP was added while the oxidation was proceeding. The addition of cyanide caused the rate to fall to 8.6 nmoles O_2/min.mg protein. The addition of ATP then caused the rate to rise to 10.8. The addition of SHAM then caused the rate to fall to 5.7.

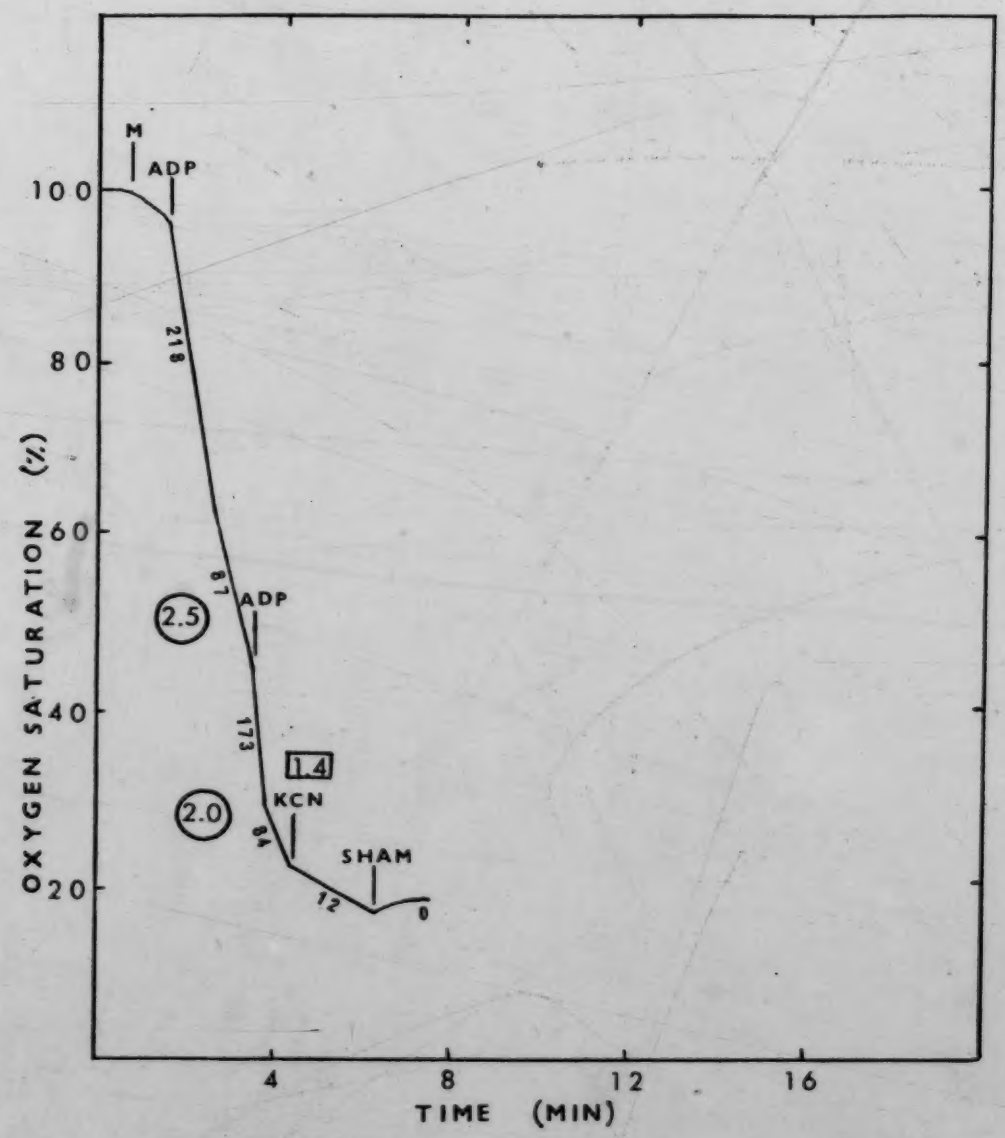

M
ADP
100
80
60
40
20
OXYGEN SATURATION (%)
218
87
ADP
2.5
173
1.4
KCN
2.0
84
SHAM
12
0
4
8
12
16
TIME (MIN)

FIGURE 11. Oxidation of NADH by pea cotyledon mitochondria.

Mitochondria from 6 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The concentration of the NADH was approximately 1 mM. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

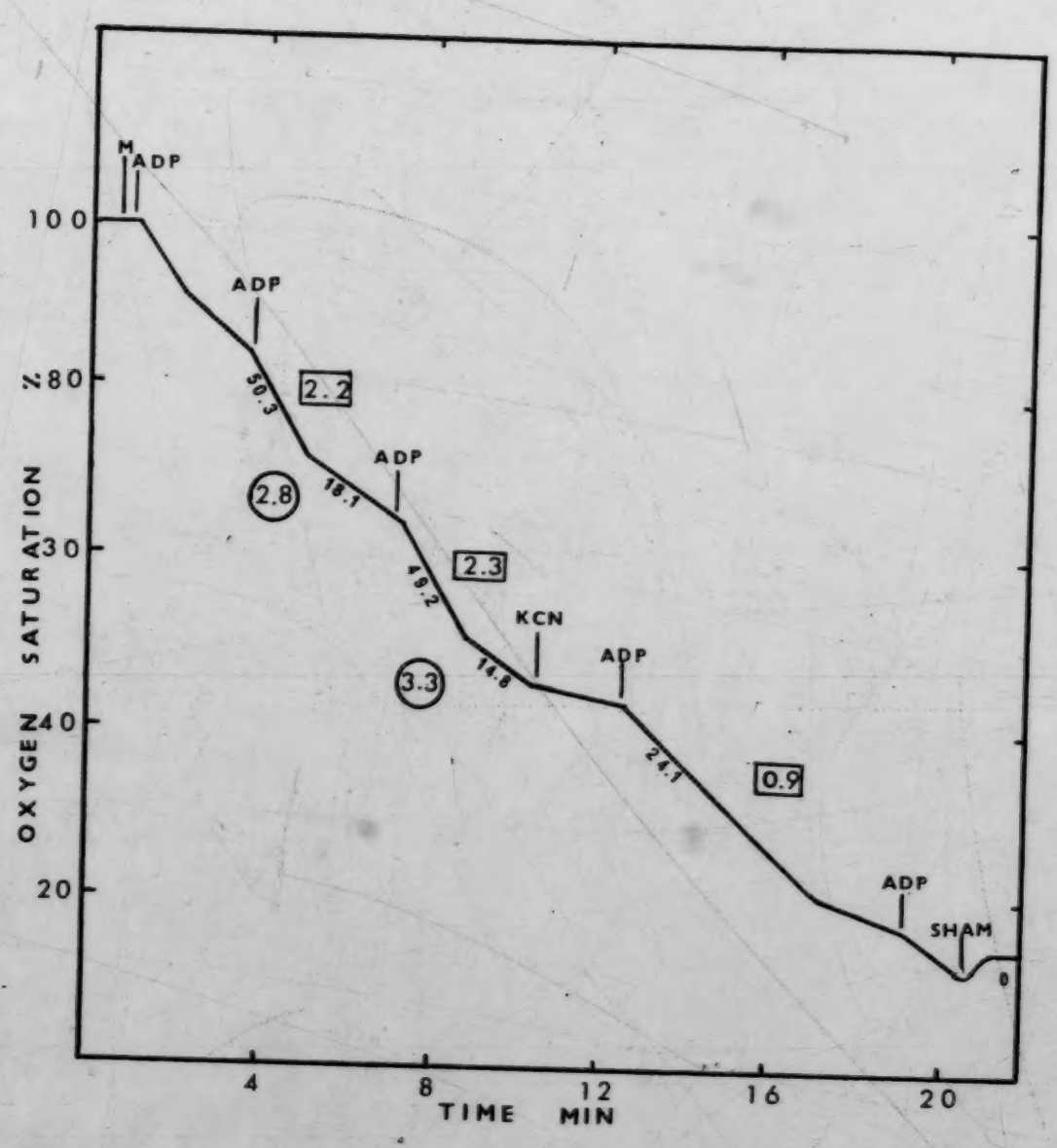

M
ADP
ADP
ADP
KCN
ADP
ADP
SHAM
50.3
18.1
19.2
14.8
24.1
2.2
2.8
2.3
3.3
0.9
0
100
80
60
40
20
OXYGEN SATURATION %
4
8
12
16
20
TIME MIN

FIGURE 12. Oxidation of citrate by pea cotyledon mitochondria.

Mitochondria from 6 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The concentration of citrate was 8 mM. The numbers alonside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

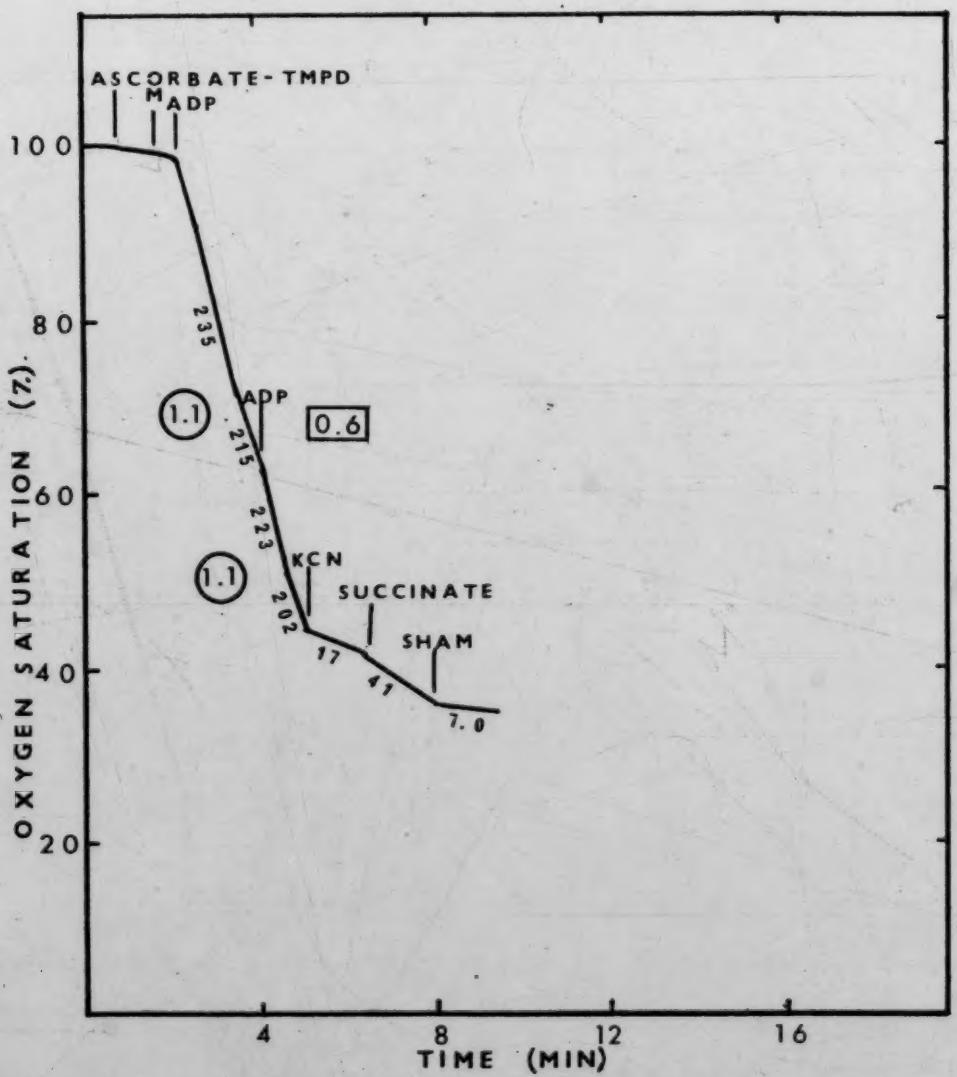

ASCORBATE-TMPD
M
ADP
100
80
60
40
20
OXYGEN SATURATION (%)
235
ADP
0.6
1.1
215
223
KCN
1.1
SUCCINATE
202
SHAM
17
41
7.0
4
8
12
16
TIME (MIN)

FIGURE 13. The oxidation of ascorbate-TMPD by pea cotyledon mitochondria.

Mitochondria were prepared from 6 day old cotyledons by the zonal technique and were assayed polarographically. (See Materials and Methods). The concentration of ascorbate was 4 mM, and the concentration of TMPD was 0.2 mM. Succinate was added to a final concentration of 8 mM. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio

7. Oxidation of Duroquinol

The oxidation of duroquinol proceeded quite rapidly and with some respiratory control (Figure 14). The ADP:O ratios indicated that the electrons were passing through the last two sites of phosphorylation. The inhibition by cyanide was almost complete and the remaining respiration was not inhibited by SHAM. In fact the addition of SHAM caused an increase in the oxygen uptake rate.

8. Oxidation of Menadiol

The auto-oxidation of menadiol (Figure 15) proceeded very rapidly, even at pH 6.0. Therefore measurements with this substrate were very difficult to make. However the addition of mitochondria increased the oxygen uptake rate above that of the auto-oxidation rate. This rate was somewhat inhibited by cyanide. The addition of SHAM did not decrease this rate but in fact temporarily stimulated it. In control experiments without mitochondria a similar effect occurred.

9. Inhibition by Rotenone

An inhibition versus concentration curve for rotenone inhibition of malate oxidation is shown in Figure 16. Increasing the concentration past 10 uM did not increase the inhibition. At this concentration about 40% of the malate oxidation was inhibited. The addition of cyanide to rotenone inhibited mitochondria caused a further reduction in the respiration rate to less than 15% of the original state 3 rate.

10. Inhibition by Chloroquine

An inhibition versus concentration curve for chloroquine inhibition

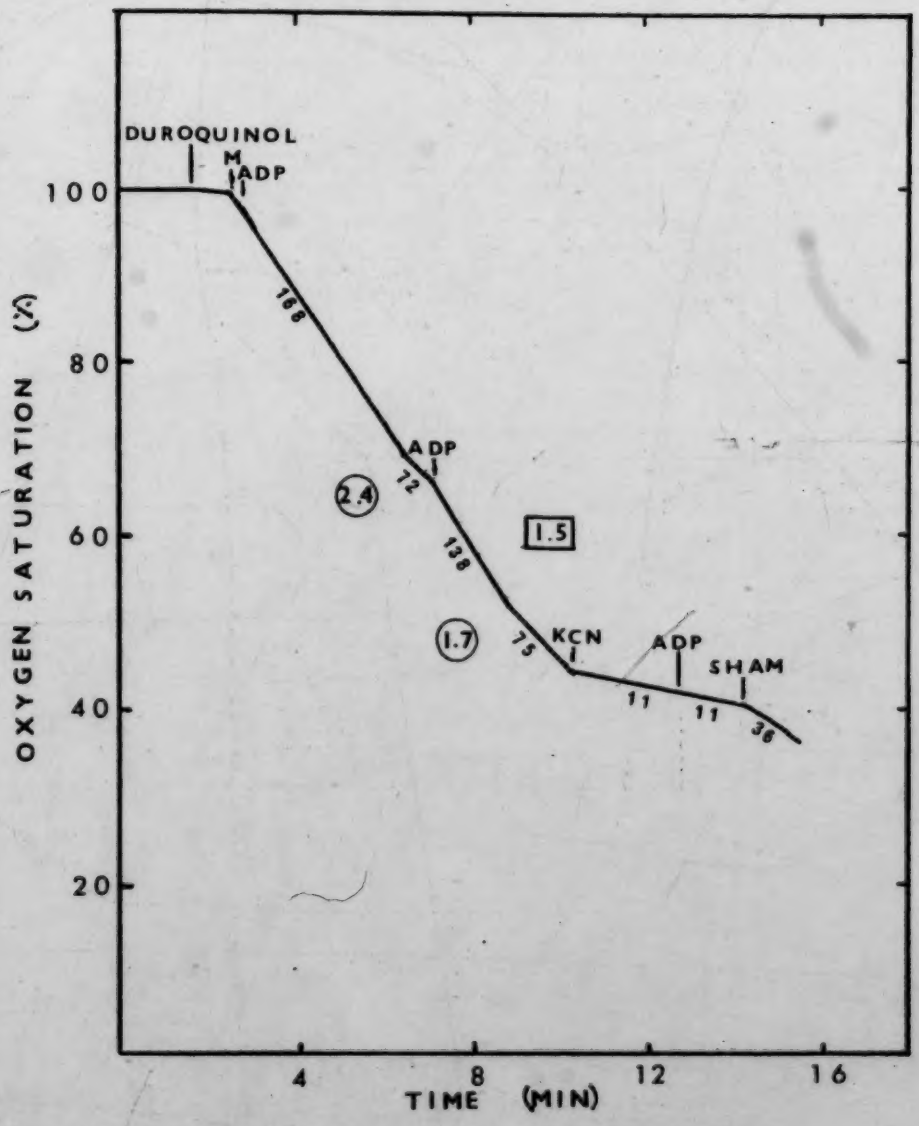

DUROQUINOL
M
ADP
100
80
60
40
20
OXYGEN SATURATION (%)
168
ADP
72
2.4
138
1.5
1.7
75
KCN
ADP
SHAM
11
11
36
4
8
12
16
TIME (MIN)

FIGURE 14. Oxidation of duroquinol by pea cotyledon mitochondria.

Mitochondria from 6 day old cotyledons were isolated by the zonal technique and assayed polarographically. (See Materials and Methods). The concentration of duroquinol was 0.3 mM. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

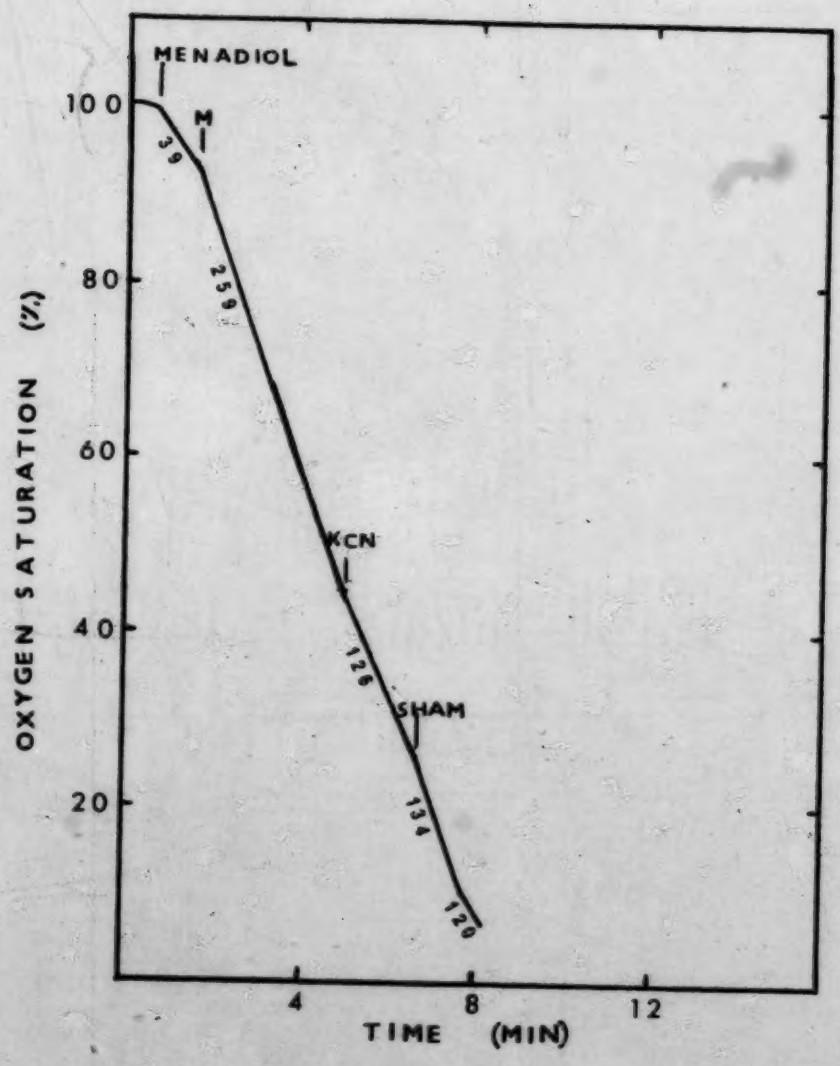

MENADIOL
M
KCN
SHAM
2.9
2.59
1.26
1.24
1.20
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
TIME (MIN)

FIGURE 15. Oxidation of menadiol by pea cotyledon mitochondria.

Mitochondria from 6 day old cotyledons were isolated by the zonal technique and assayed poarographically. (See Materials and Methods). The concentration of menadiol was 0.3 mM. The numbers alonside the traces refer to oxygen uptake in nmoles O_2/min . mg protein.

of cyanide-resistant respiration is shown in Figure 17. Half-maximal inhibition was occurred near 0.4 mM.

Chloroquine did not inhibit the state 3 respiration rate at either pH 7.2 or 8.2 when succinate was used as a respiratory substrate. However the state 4 rate was slightly accelerated at pH 7.2 and complete uncoupling occurred at pH 8.2. Chloroquine inhibition of malate oxidation was similar at pH 7.2 (Figure 19). However at pH 8.2 the situation was different. Chloroquine at 0.1 mM inhibited the malate oxidation by 30% while almost complete inhibition occurred at 3mM.

11. Reproducibility of Respiratory Measurements

Phillips (81) from this laboratory, has shown that oxygen uptake measurements with the apparatus and procedures used in this study are reproducible to within $\pm$ 5%. However in the hands of the present author the reproducibility of the respiration rate measurements was probably in the vicinity of $\pm$ 10%. Drift by the recorder and oxygen probe was usually about 1 to 2% per run and was probably not a major source of error. However the Hamilton syringes used to inject the mitochondria probably had a reproducibility of less than 5%. This coupled with small changes in the volume of the reaction vessel as the probe was inserted and other miscellaneous sources of error probably accounted for the relatively large variability. The cyanide resistant respiration when expressed as a percentage of the total respiration varied very little between runs of the same preparation, usually less than 2 to 3 percent. However differences of up to 8% occurred between runs of different preparations.

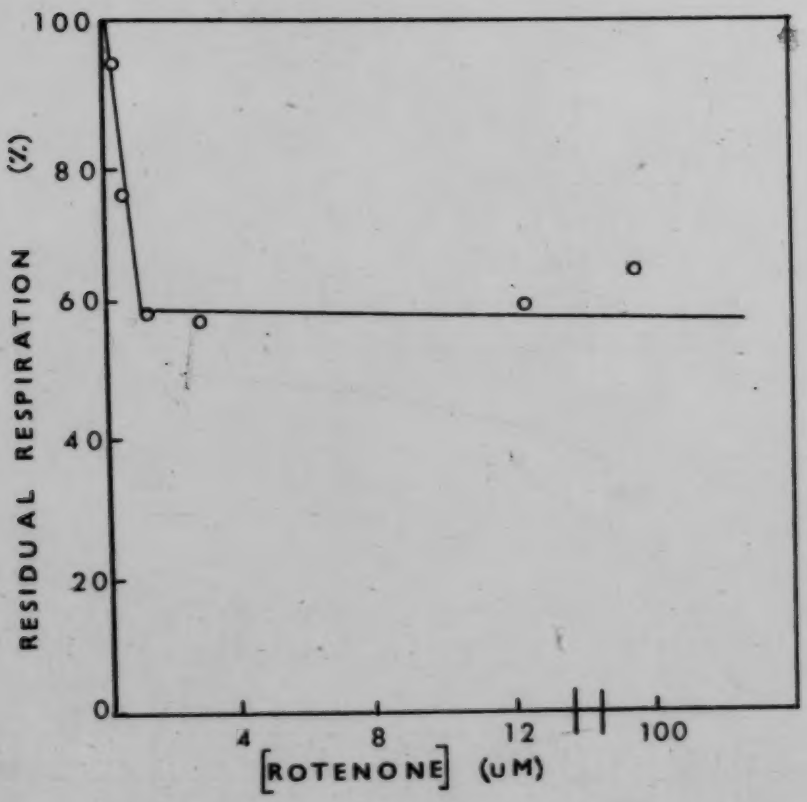


FIGURE 16. The inhibition of malate oxidation by rotenone.

Mitochondria were isolated from 6 day old cotyledons by the zonal technique and were allowed to oxidize malate. Rotenone, at the indicated concentrations, was added during the second period of state 3 respiration.

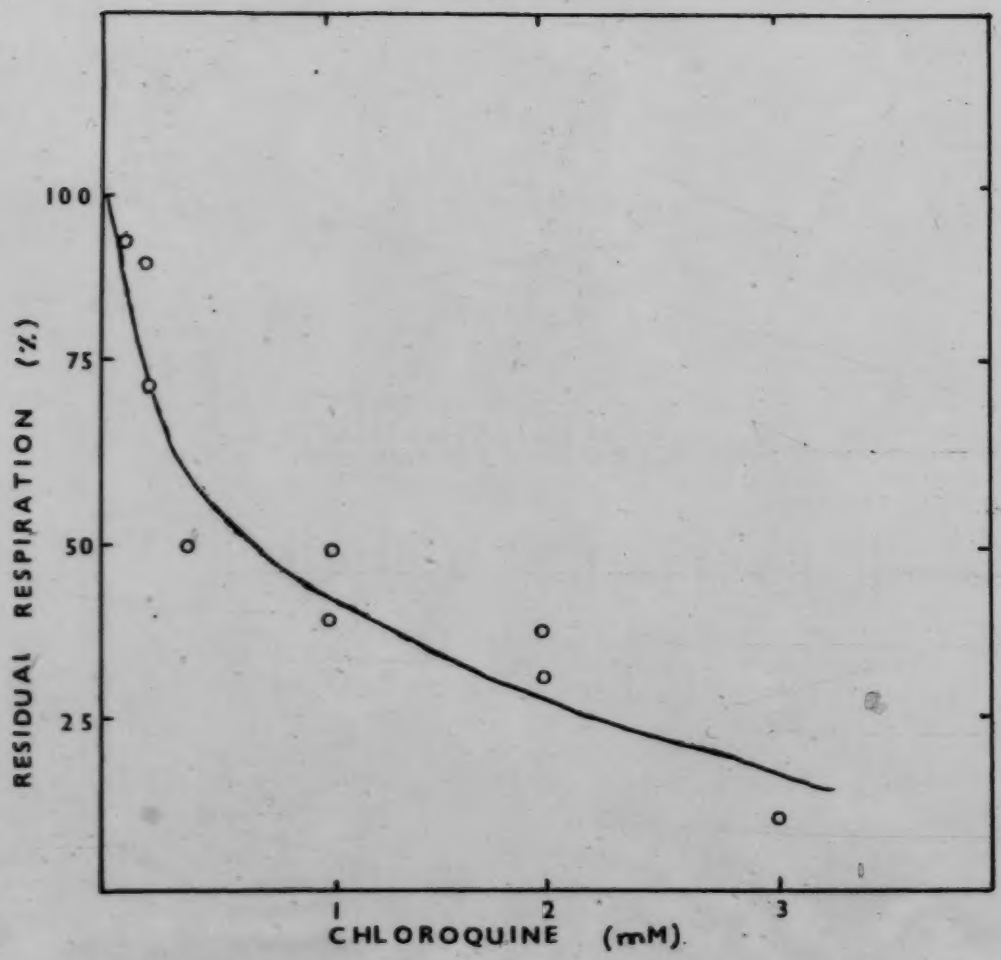

100
75
50
25
RESIDUAL RESPIRATION (%)
1
2
3
CHLOROQUINE (mM)

FIGURE 17. Inhibition of cyanide-resistant respiration by chloroquine.

Mitochondria from 6 day old cotyledons were prepared by the zonal procedure and were assayed polarographically. (See Materials and Methods). The respiratory substrate was succinate. KCN was added following the second period of state 3 respiration and following this chloroquine was added.

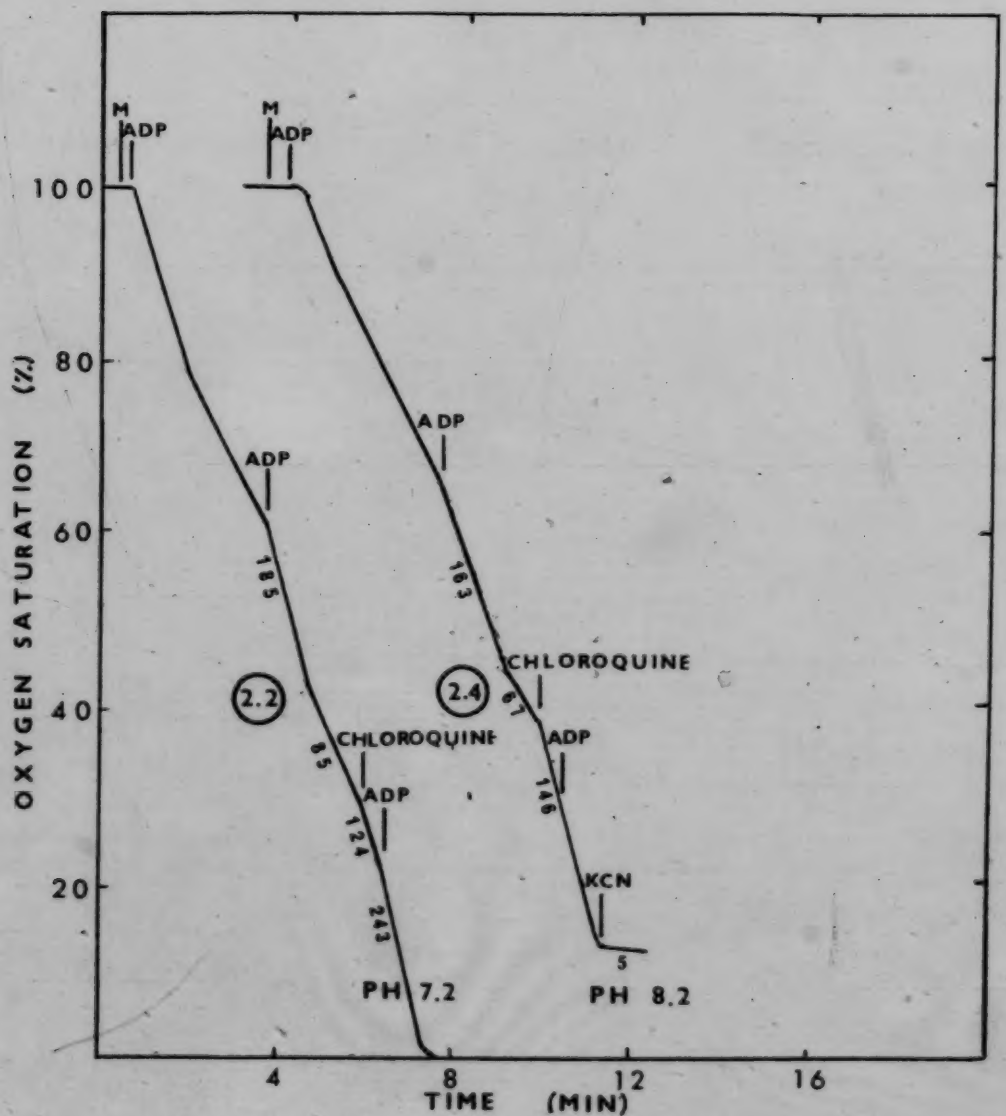

M
ADP
100
80
60
40
20
OXYGEN SATURATION (%)
ADP
185
2.2
CHLOROQUINE
ADP
124
243
PH 7.2
M
ADP
ADP
183
2.4
CHLOROQUINE
ADP
149
XCN
5
PH 8.2
4
8
12
16
TIME (MIN)

FIGURE 18. Inhibition of succinate oxidation by chloroquine.

Mitochondria were prepared from 6 day old cotyledons by the zonal technique and were assayed polarographically. (See Materials and Methods). The final concentration of chloroquine was 3 mM. The numbers alonside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

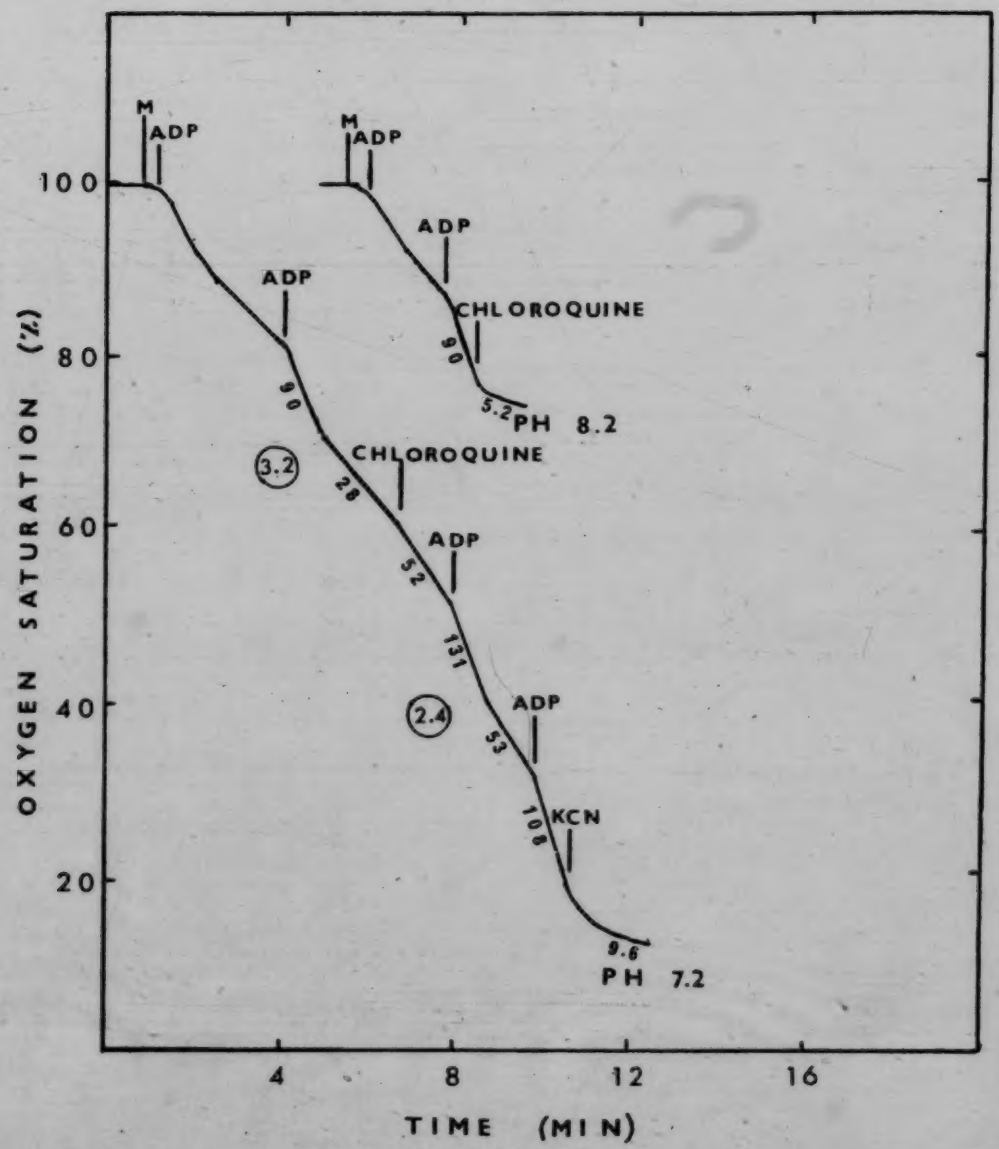

M
ADP
M
ADP
ADP
ADP
CHLOROQUINE
90
5.2
PH 8.2
3.2
90
CHLOROQUINE
28
ADP
52
131
2.4
ADP
53
KCN
108
9.6
PH 7.2
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 19. Inhibition of malate oxidation by chloroquine.

Mitochondria were isolated from 6 day old cotyledons by the zonal technique and were assayed polarographically. (See Materials and Methods). The final concentration of chloroquine was 3 mM. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein.

Different lots of seed seemed to vary with regards to cyanide-resistant respiration. However this hypothesis was not investigated rigorously.

D. The Redox State of Coenzyme Q

Experiments of the type shown in Figure 20 were used to determine the redox state of the Coenzyme Q under different experimental conditions. The spectra shown in Figure 20 illustrate the characteristic absorption peak of ubiquinone at 275 mm in the oxidized form and the shift in this peak to 290 nm on reduction. The strong absorbance at the lower wavelengths probably arises from contaminating lipids in the preparation.

The redox state of Coenzyme Q under different experimental conditions is given in Table II. The ubiquinone was considered to be 100% oxidized in the case where no respiratory substrate was present in the assay medium. When this is taken as 100% the ubiquinone was 29% reduced in state 3 and 52% reduced in state 4. In the presence of cyanide 48% (in state 3) and 53% (in state 4) of the Coenzyme Q was reduced. The addition of SHAM to the mitochondria in either state 3 sr State 4 led to a slight oxidation of the ubiquinone.

It was also possible to determine the ubiquinone content of the mitochondria as a result of these experiments. Using a value of 12,250 for the molecular extinction coefficient (10), values of 1.9 nmoles ubiquinone/ mg protein and 2.4 nmoles ubiquinone/mg protein were obtained.

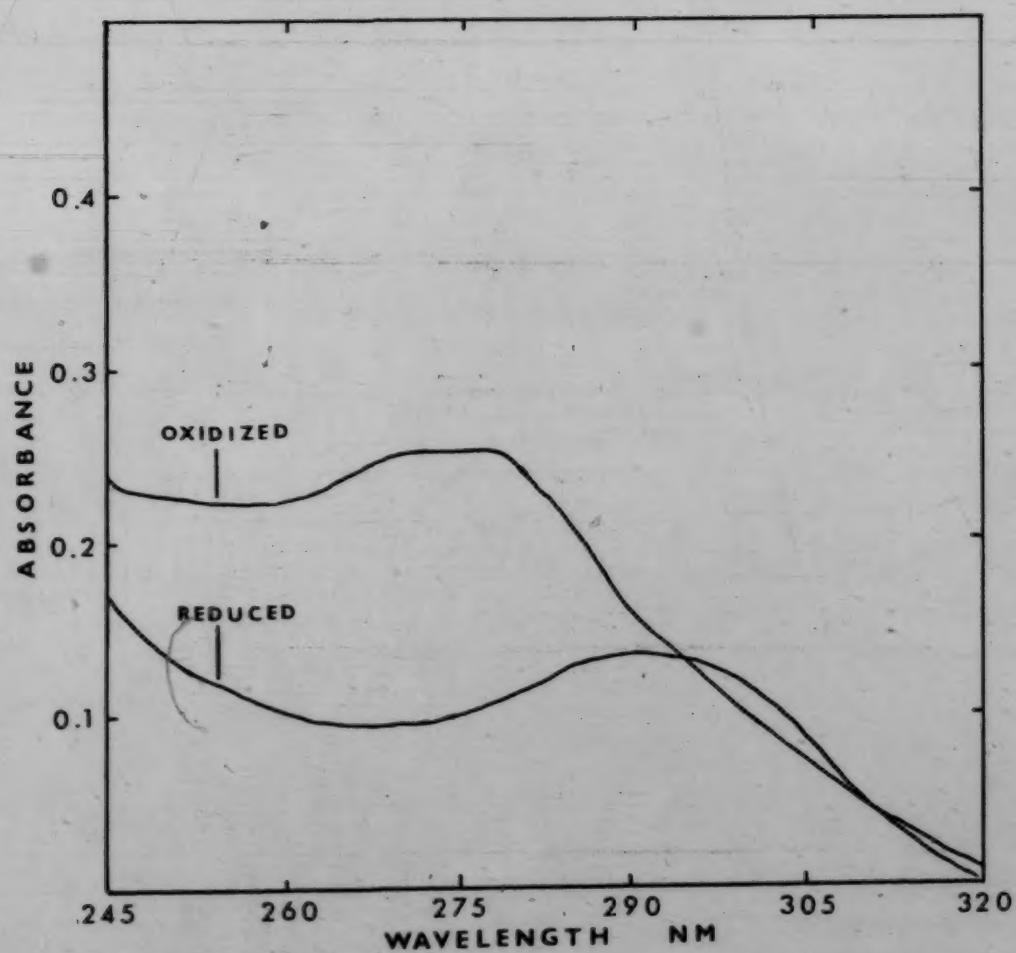

0.4
0.3
0.2
0.1
ABSORBANCE
OXIDIZED
REDUCED
245
260
275
290
305
320
WAVELENGTH NM

FIGURE 20. The absorption spectrum of ubiquinone obtained by petroleum ether extraction.

Washed mitochondria were incubated in the standard polarographic assay medium for three minutes. A mixture of petroleum ether and methanol (60:40 v/v) was then added. The extract was washed once with 95% methanol, the petroleum ether was removed and the residue was dissoved in an ethanol: heptane mixture (4:1 v/v). Reduction was achieved by the addition of a few grains of sodium borohydride.

TABLE II

The Redox State of Ubiquinone Under Various Experimental Conditions.

Mitchondria were incubated for 3 minutes in a medium containing 0.3 M mannitol, 5 mM phosphate, 5 mM $MgCl_2$, 8 mM succinate, 0.75 mg/ml BSA, and 50 mM TES with the pH adjusted to 7.2 by Tris. The reaction was terminated by the addition of the petroleum ether extracting solution.

The absorbance spectrum of the extracted ubiquinone was then determined. KCN and SHAM were added to give a concentration of 0.2 mM and 1 mM respectively. The concentration of ADP for the state 3 condition was 1 mM. The values are the average of duplicate runs.

	Preparation									
	1		2		3		4		5	
Assay Condition	A*	%**	A	%	A	%	A	%	A	%
no succinate	.19	0	.105	0	.21	0	.20	0	.09	0
state 3	-	-	-	-	.15	29	-	-	.16	28
state 4	-	-	-	-	.10	52	.09	55		
state 4 + KCN	.07	73	.05	52	.11	48	-	-	-	-
state 4 + KCN + SHAM	.09	53	.05	52	.15	29	-	-	-	-
state 4 + SHAM	-	-	-	-	-	-	.13	35	-	-
state 3 + SHAM									.07	22

*Absorbance at 275, oxidized minus reduced

**% reduction

E. The Effect of Ubiquinone Extractions on Mitochondrial Reactions

It was possible through repeated pentane extractions to remove much of the Coenzyme Q from the mitochondria. The absorption spectrum of the pentane extracts is shown in Figure 21, and the oxidation of succinate by such a preparation is shown in Figure 22. The preparation in which there was absolutely no respiration was not achieved. However up to 90% of the activity of the lyophilized mitochondria was lost. NADH oxidizing activity was lost more rapidly than succinate oxidizing activity (Table III). Much of the activity could be restored again through the reincorporation of Coenzyme Q_6 (Figure 23). Some activity could also restored by direct addition of duroquinone to the extracted mitochondria. Duroquinone restored NADH oxidation to a much greater extent than it did succinate oxidation (Figure 24). Activity could also be restored through the direct addition of Coenzyme Q_6 to the reaction medium (results not shown) but the activity of such a preparation did not exhibit linear uptake of oxygen. The addition of cytochrome c was beneficial in restoring the oxidizing activity.

Despite repeated attempts, it was impossible to obtain a reconstituted mitochondrial preparation that exhibited cyanide resistant respiration. The cyanide resistant respiration survived freezing but it did not survive the lyophilization procedure (Figure 25).

F. The Effect of Cotyledon Age on Mitochondrial Oxidations

The effect of seedling age on the respiratory activity of the mitochondria and on the amount of cyanide-resistant respiration is shown in Table IV. Mitochondria from pea cotyledons that have imbibed for

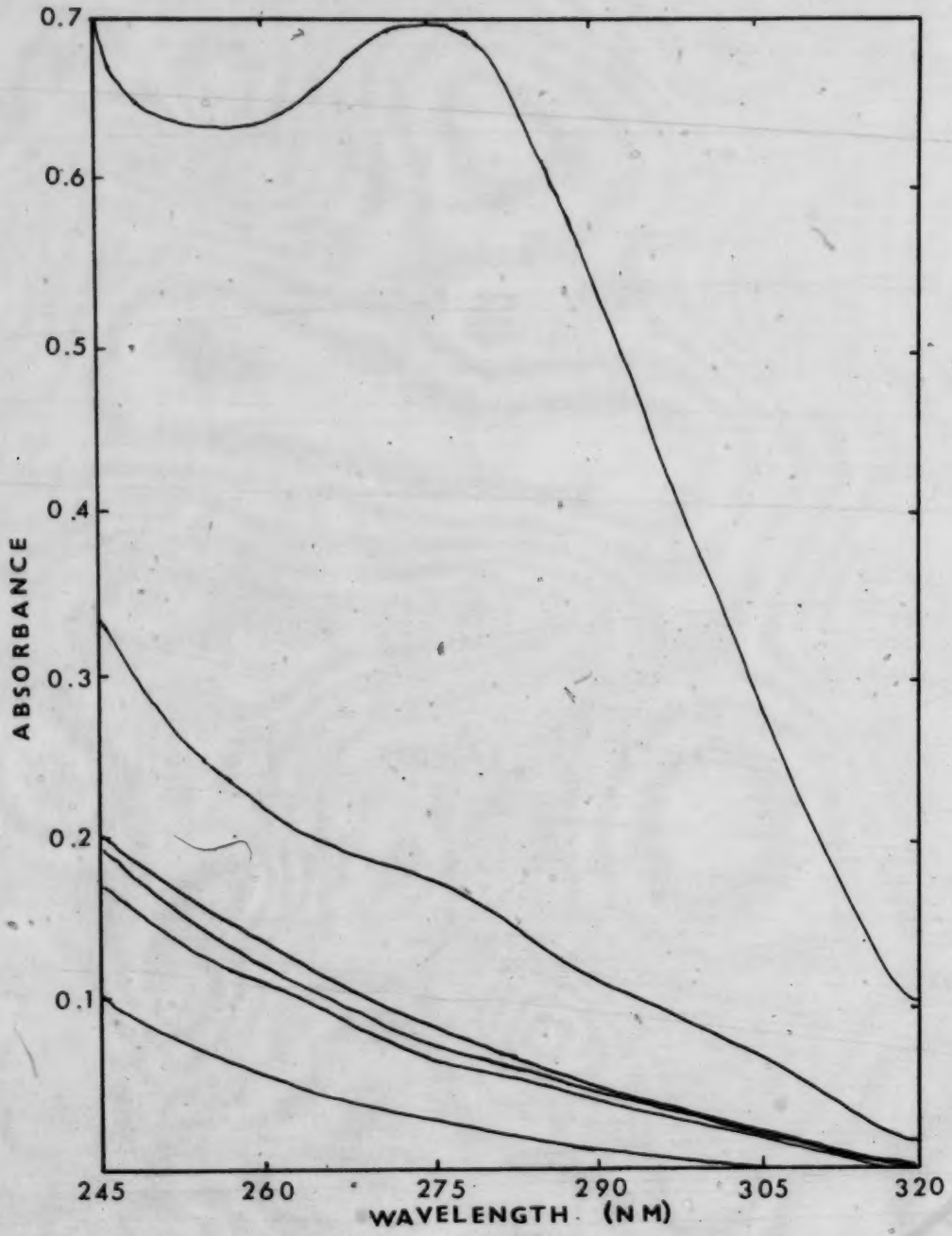

0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
245
260
275
290
305
320
WAVELENGTH (NM)

FIGURE 21. The absorption spectra of a series of pentane extractions of lyophilized mitochondria.

Washed mitochondria were lyophilized and then homogenized repeatedly with 5 ml aliquots of n-pentane. The absorption spectra of these extracts was taken. The upper curve is the spectrum of the first extract, and the others follow consecutively.

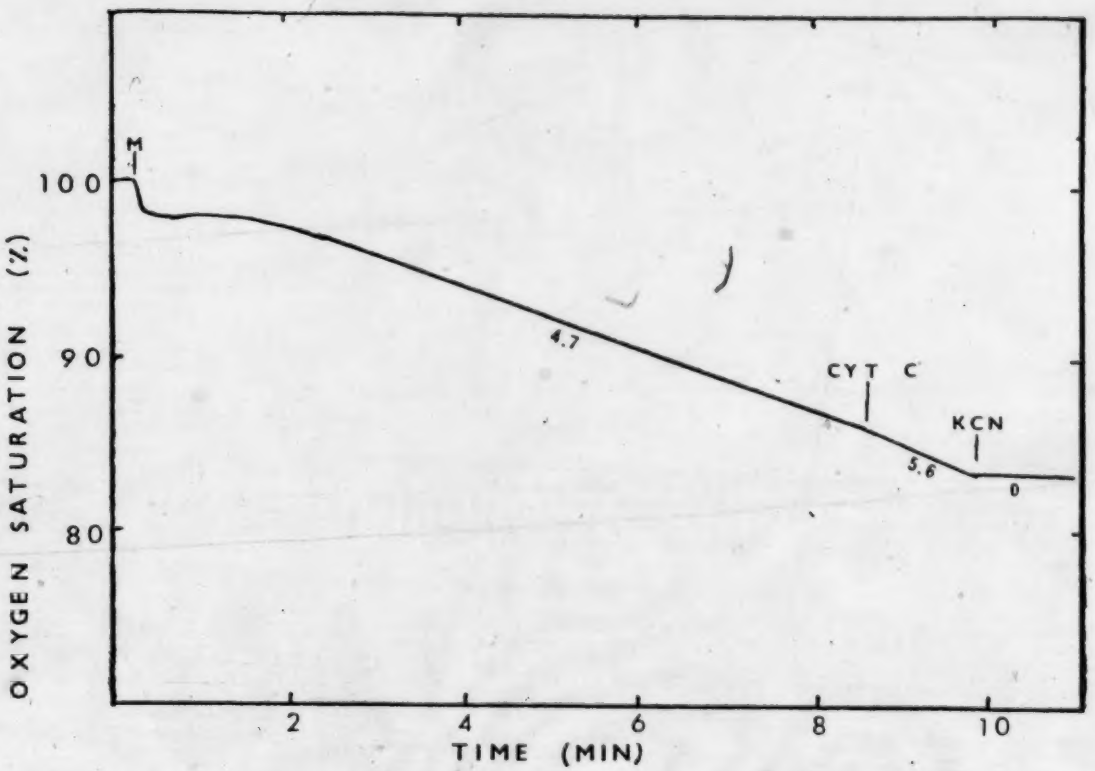


FIGURE 22. The oxidation of succinate by pentane extracted mitochondria.

Washed mitochondria were lyophilized and then extracted five times with pentane. The pentane was removed and the residue was suspended in mitochondrial suspending medium and assayed in the usual manner. (See Materials and Methods). The numbers alonside the trace refer to oxygen uptake in nmoles O_2/min . mg protein.

TABLE III

The Effect of Pentane Extractions on Mitochondrial Oxidations.

Lyophilized mitochondria were extracted with pentane a number of times. After the appropiate number of extractions the pentane was removed. The mitochondria were resuspended in the usual suspending medium and were assayed polarographically. (See Materials and Methods).

Treatment	Oxidations Rate (nmoles O_2/min . protein	
	Succinate	NADH
Lyophilized Mitochondria	26.8	25.2
2X extracted	10.1	4.3
4X extracted	3.8	2.1

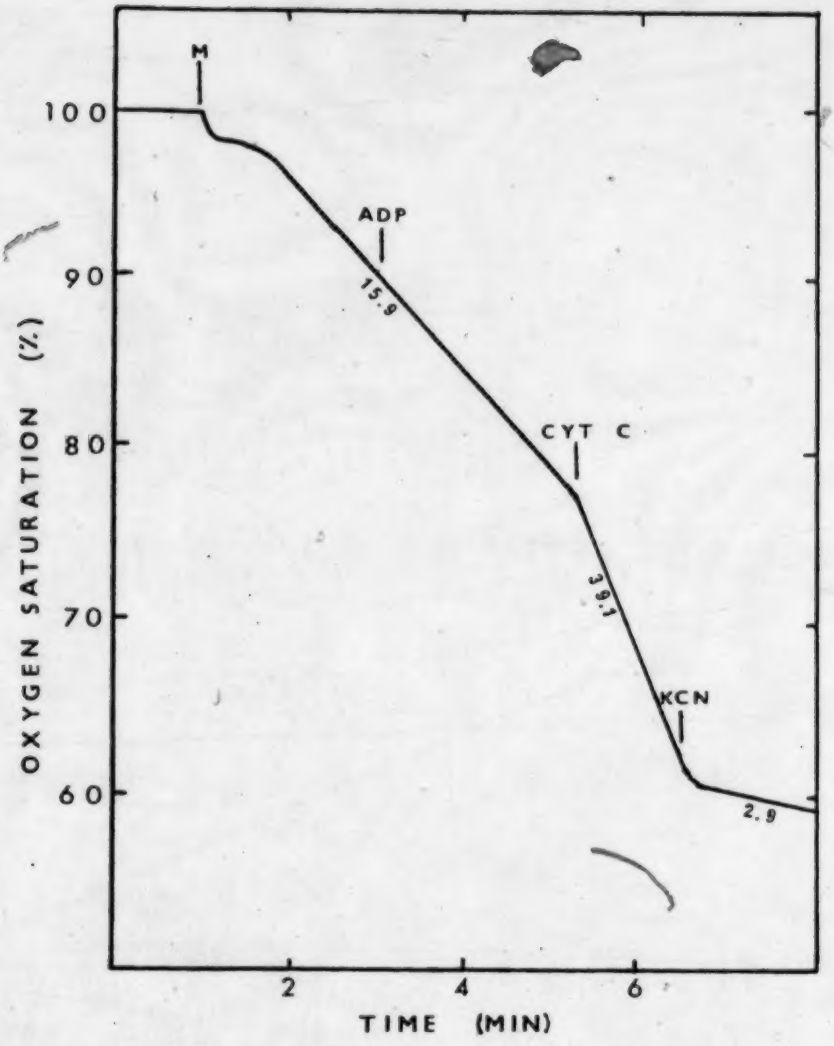

M
ADP
CYT C
KCN
15.9
3.9.1
2.9
100
90
80
70
60
OXYGEN SATURATION (%)
2
4
6
TIME (MIN)

FIGURE 23. Oxidation of succinate by pentane extracted and CoQ_6 reincorporated mitochondria.

Lyophilized mitochondria were extracted with pentane 5 times. Coenzyme Q_6 was then reincorporated into the mitochondria using a small amount of pentane. The pentane was removed and the mitochondria were suspended and assayed in the usual manner. (See Materials and Methods). Cytochrome c was added to a final concentration of 0.2 umoles/ml. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein.

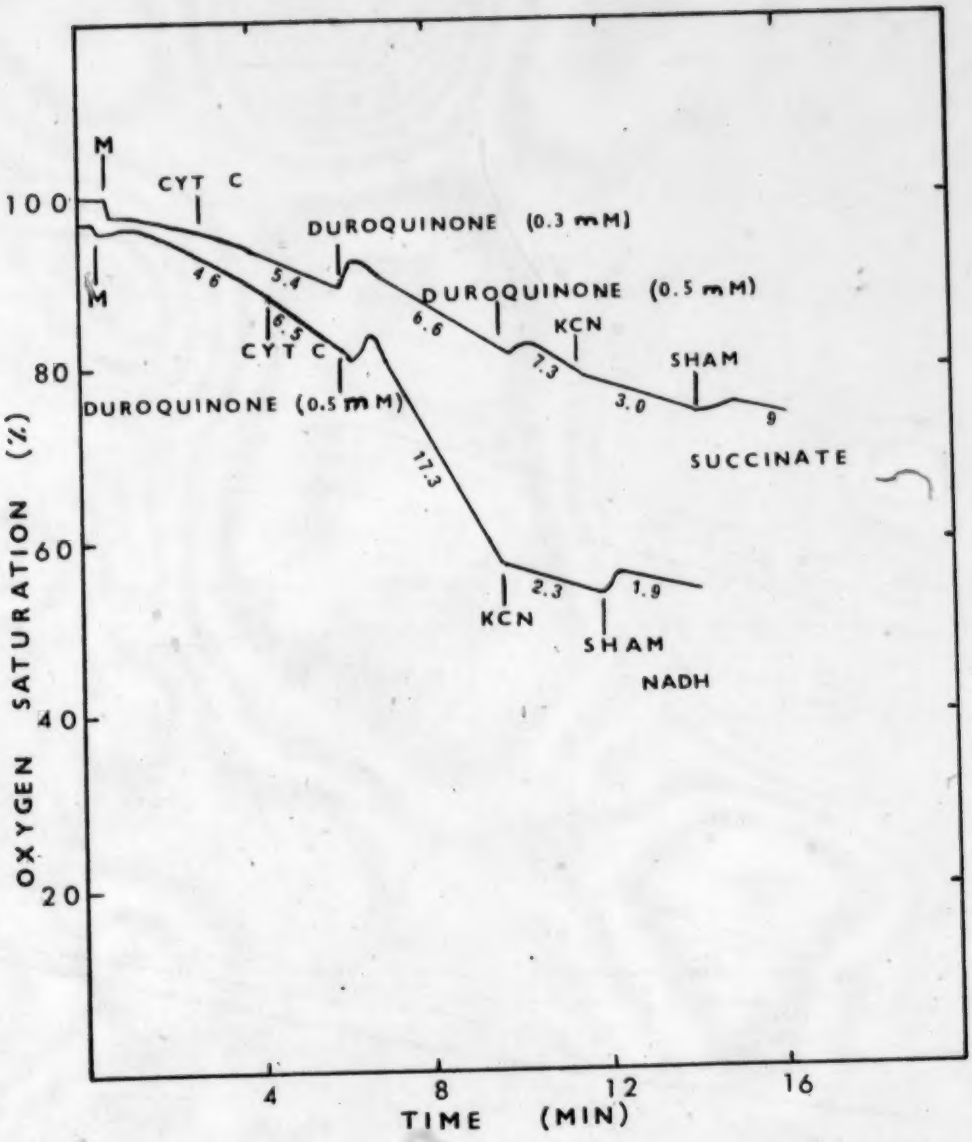

M
CYT C
DUROQUINONE (0.3 mM)
DUROQUINONE (0.5 mM)
KCN
SHAM
SUCCINATE
M
CYT C
DUROQUINONE (0.5 mM)
KCN
SHAM
NADH
4.6
5.4
6.5
6.6
7.3
3.0
9
17.3
2.3
1.9
100
80
60
40
20
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 24. Reconstitution of electron transport in pentane extracted mitochondria by duroquinone.

Washed mitochondria were lyophilized and then extracted with pentane 5 times. The pentane was removed and the mitochondria were resuspended in the usual suspending medium and assayed in the usual manner. (See Materials and Methods). The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein.

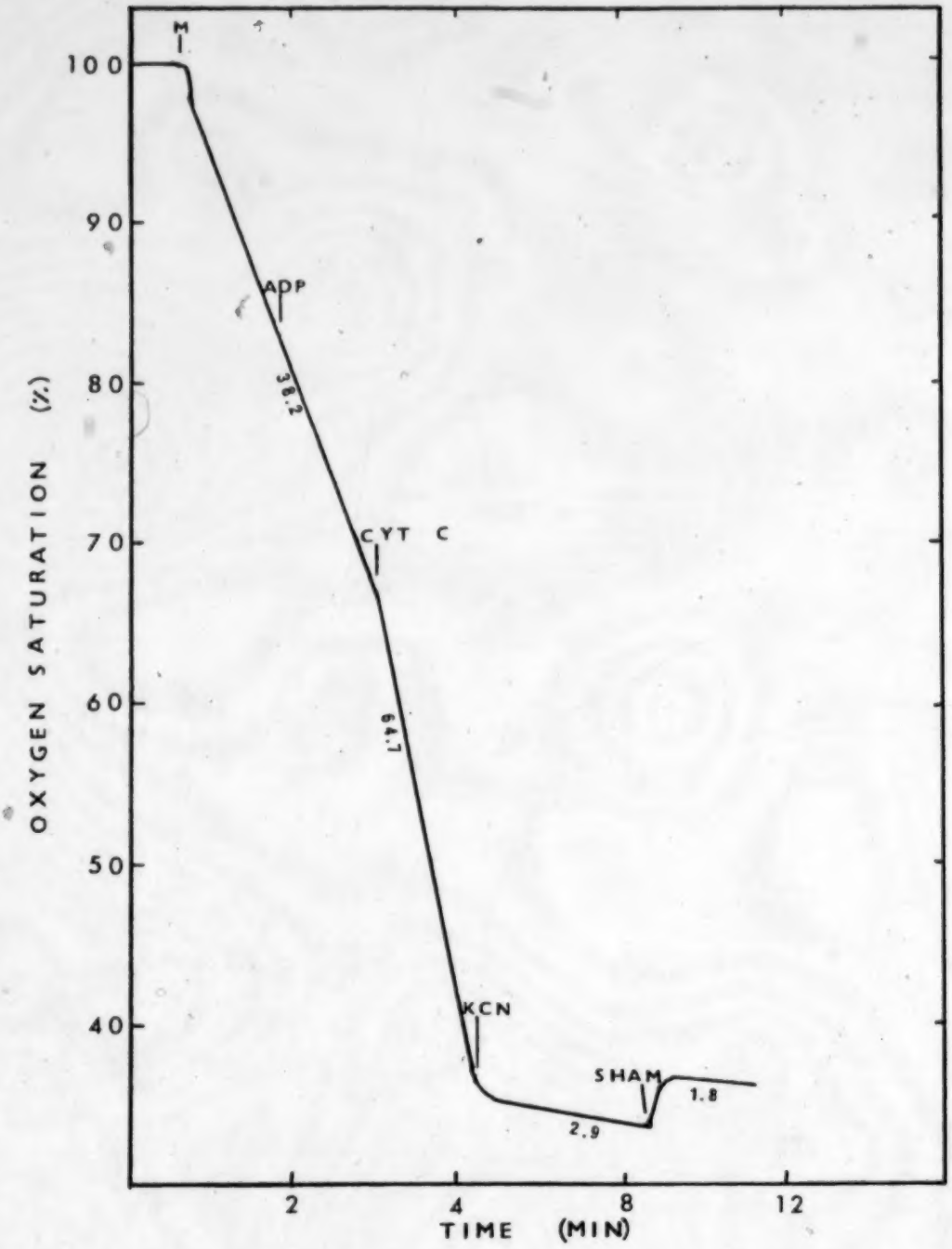

M
ADP
CYT C
KCN
SHAM
2.9
1.8
OXYGEN SATURATION (%)
100
90
80
70
60
50
40
2
4
8
12
TIME (MIN)

FIGURE 25. Oxidation of succinate by lyophilized mitochondria.

Washed mitochondria were prepared from 6 day old cotyledons and were suspended in 0.15 M KCl. They were then lyophilized for 6 hours. Following this they were suspended in the usual suspending medium and assayed in the usual manner. (See Materials and Methods). Cytochrome c was added to a final concentration of 0.2 umoles/ml. The numbers alongside the traces refer to oxygen uptake in nmoles O_2/min . mg protein.

TABLE IV

Respiration and Cyanide Resistance in Mitochondria Isolated from Germinating Pea Cotyledons.

The procedures used are those described in Materials and Methods section. The numbers given are median values obtained with several runs from two or more different preparations. The average deviation between runs of the same preparation was ± 10% and ± 20% between runs of different preparations. The average deviation of the cyanide resistant respiration was ± 3% between runs of the same preparation and up to ± 8% between runs from different preparations.

		Cotyledon Age[a] (days)									
Substrate	Parameter	0.2	1	2	3	4	5	6	7	8	10
Succinate (8 mM)	Oxygen uptake, state 3[b]	56.8	124	114	143	272	291	330	210	186	160
	Respiratory control ratio	2.6	3.5	1.9	3.4	4.3	3.5	3.7	2.6	3.4	2.3
	ADP:O Ratio	1.3	1.5	1.4	1.6	1.5	1.5	1.5	1.5	1.2	1.3
	Cyanide-resistant rate[b]	0	0	12	13	30	32	38	35	22	17
	% Cyanide resistant	0	0	11	9	11	11	14	17	12	11
α-Keto-gularate (8 mM)	Oxygen uptake, state[b]	38	28	65	61	128	113	143	112	104	90
	Respiratory control ratio	2.5	5.4	3.7	11.7	5.8	5.3	7.6	6.0	7.7	5.3
	ADP:O Ratio	2.2	2.8	3.0	3.4	3.6	3.5	3.3	3.2	3.2	2.5
	Cyanide-resistant rate[b]	0	3.7	9.8	10	33	27	40	31	24	14
	% Cyanide resistant	0	7.3	15	16	26	33	28	29	23	15
L-malate (10 mM)	Oxygen uptake, state 3[b]	11	7.4	47	79	114	117	114	95	87	72
	Respiratory control ratio	4.0	3.0	3.9	5.4	3.5	5.2	5.3	5.0	3.7	4.3
	ADP:O Ratio	1.9	1.9	2.2	2.5	2.2	2.5	2.5	2.3	2.3	1.8
	Cyanide-resistant rate[b]	0	0	8.7	15	29	30	32	27	15	12
	% Cyanide resistant	0	0	19	19	25	26	28	28	17	16

[a] Time measured from the onset to imbibition to the time of harvest.

[b] nmoles O_2/min/mg protein.

only a few hours have very low rates of oxidation and no cyanide resistance. However after 24 hours of imbibition the succinoxidase systems seems to be well developed. Relatively high rates of oxygen uptake were obtained and there was good respiratory control. In fact respiratory control at this stage with succinate was often better than at later stages of development. The malate and α-ketoglutarate oxidizing systems developed more slowly. Little development took place until the second day of germination. At this time both the oxidation rate and the respiratory control improved. From the second day until the sixth day oxidation rates for malate, α-ketoglutarate, and succinate all increased. The most substantial increases occurred between the second and fourth days. The period between the fourth and sixth days was more of a plateau period with increases being smaller. After the sixth day respiratory rates declined. The highest degree of respiratory control was also obtained near the fourth day, but relatively good respiratory control could be obtained at all stages of germination.

The cyanide resistant respiration did not appear until the second day of germination. After this time, it increased both in absolute amounts and in proportion to the total respiration rate. It reached a maximum near the sixth day of germination and declined after that somewhat.

G. Effect of Alteration of Germination Conditions

1. Inclusion of Chloramphenicol

Inclusion of chloramphenicol in the germination medium at the concentration used in this study (0.5 mg/ml) did not alter greatly the

respiratory parameters studied, even though the growth of peas was inhibited. The respiration rate was slightly less and the respiratory control ratios slightly lower, but the amount of cyanide resistance was not altered.

2. Inclusion of Azide

The inclusion of azide at 10^{-4}M or 10^{-6}M did not affect the amount of cyanide resistance. However the respiratory control ratios were much lower and the mitochondria seemed to deteriorate quickly after isolation. After 30 minutes no respiratory control could be observed. A similar experiment was conducted with cyanide. With 10^{-6} M cyanide in the germination medium, there was no discernible effects on the mitochondria. However at 10^{-5} M cyanide no germination occurred.

3. Effect of Light

Mitochondria isolated from peas that had been grown in continuous light for 6 or 9 days did not behave differently than peas that had not received light, with the exception that at 9 days the respiratory control ratios were reduced by a factor of 2 or 3.

4. Effect of Ethylene

Initial experiments using a crude ethylene treatment showed that ethylene did indeed alter the amount of cyanide resistance. Washed mitochondria from 6 day old peas grown in closed respiratory jars in an atmosphere containing 100 ppm ethylene had between 25 and 30% of the succinate oxidation rate resistant to cyanide. In other respects the mitochondria behaved normally. The more rigorous flow through treatment

in which purified air was used as a carrier for the ethylene and after which the mitochondria were isolated zonally yielded similar results (Figure 26).

The concentration of ethylene required to achieve this effect was not investigated rigorously. However in one experiment peas were grown for 5 days in a closed respiratory jar initially containing only oxygen. Enough ethylene was produced by the peas themselves to produce typical ethylene injury symptoms, such as growth inhibition, and radial expansion of the young shoot. Mitochondria isolated from these peas again had high amounts of cyanide resistant respiration.

Another experiment indicated that this was not a direct effect of the oxygen. Cleaned cotyledons from 3 day old peas were divided into two lots. One lot was sealed in a respiratory jar with 100 ppm ethylene in a background of 100% oxygen. The other was continously flushed with oxygen. Mitochondria from both lots were isolated 24 hours later. Mitochondria from both lots were isolated 24 hours later. Mitochondria from the ethylene treated peas had approximately 25% of their succinate oxidation resistant to cyanide, while the peas that received 100% oxygen had only 15% of their succinate oxidation resistant to cyanide. Cotyledons treated with 100 ppm ethylene in a purified air back ground also had only 15% of the succinate oxidation resistant to cyanide, indicating the oxygen and ethylene treatment was synergistic.

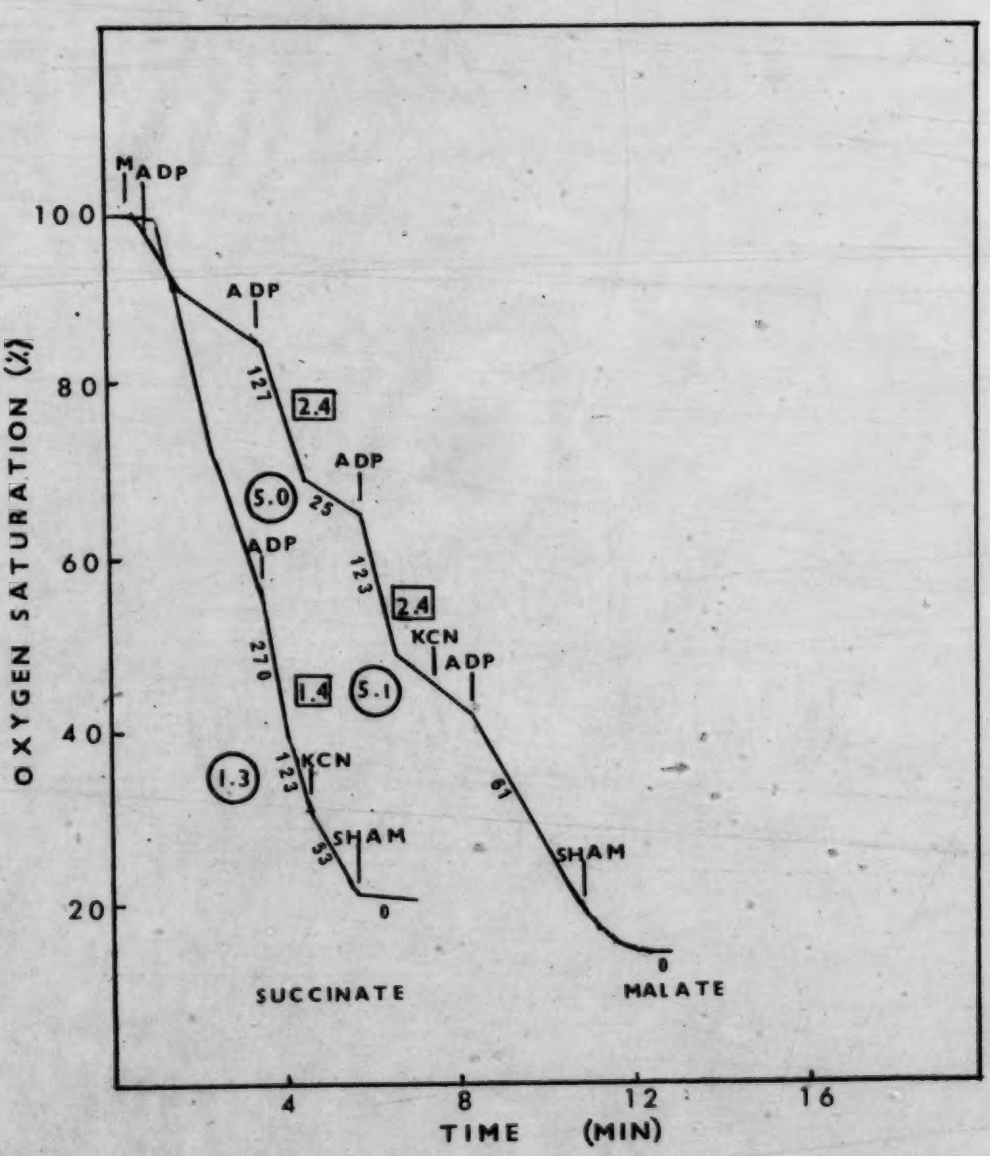

M
ADP
100
ADP
80
127
2.4
ADP
5.0
25
ADP
60
123
2.4
KCN
ADP
270
1.4
5.1
40
1.3
KCN
123
61
SHAM
53
SHAM
20
0
0
SUCCINATE
MALATE
OXYGEN SATURATION (%)
4
8
12
16
TIME (MIN)

FIGURE 26. The effects of an ethylene atmosphere during germination on mitochondrial reactions.

Peas were germinated in a container through which purified air containing 112 ppm ethylene was passed continuously. After 6 days the peas were harvested and mitochondria were isolated using the zonal technique. They were assayed in the usual manner. (See Materials and Methods). The numbers alonside the traces refer to oxygen uptake in nmoles O_2/min . mg protein. The circled numbers refer to the RCR and the numbers in rectangles to the ADP:O ratio.

IV. DISCUSSION

A. Morphological Development of the Peas

The rates at which various germination processes occur in peas and other plants are dependent on a variety of internal and external conditions. Temperature is an especially important factor. Therefore in order to correlate results between laboratories, it is necessary to have criteria other than chronological age for identifying developmental stages.

The morphological development of the peas in this study proceeded more rapidly than in the study done by Bain and Mercer (45). Phase 1 as described by them was complete at the end of the second day, phase 2 extended from the second to the fifth day, and phase 3 extended from the sixth day onward.

B. Mitochondrial Integrity and Purity

Many traditional tests of mitochondrial integrity and purity, such as the degree of respiratory control, used with animal mitochondria cannot be successfully applied to plant mitochondria. The presence of alternate non-phosphorylating pathways, and other additional features in plant mitochondria could lead to erroneous results. The tests used the present study were suggested in a recent review of electron transport in plant mitochondria (77) as alternatives to tests traditionally used with animal mitochondria.

The basis of the succinate-cytochrome c reductase test is the fact that externally added cytochrome c cannot pass through the outer mitochondrial membrane (27). It therefore cannot be reduced by the succinate dehyrogenase system of the inner membrane. As a result the test is a

measure of the fraction of the mitochondria having damaged outer membranes. In this study succinate dehydrogenase cytochrome c reductase activity was low for both the washed and zonally isolated mitochondria. It was increased greater than fifteen fold when the mitochondria were placed in a hypotonic medium. It seems reasonable to conclude therefore that only a small fraction of the mitochondria in either preparation had damaged external membranes. The fraction having damaged membranes was slightly less in the case of the zonally isolated mitochondria.

It has been suggested that the presence of antimycin-A-resistant NADPH cytochrome c reductase activity indicates microsomal contamination (77). However this test has not been widely used, perhaps because of its low sensitivity. In this study the activity was low and somewhat difficult to measure in both preparations. It was somewhat lower in the case of the zonally isolated mitochondria, indicating perhaps that this was a somewhat purer preparation. The identity of the antimycin-A-sensitive activity is not known. It may be extra-mitochondrial in nature since its activity is less in the case of the zonally isolated mitochondria. However an extra-mitochondrial site sensitive to antimycin is not known. On the whole it would seem that the above test is not a good test to measure the purity of a mitochondrial preparation.

The most reliable measure of mitochondrial purity would still seem to be electron microscopy (77). Hamman (40) used electron microscopy to show that mitochondria isolated by the zonal technique used in this study, produced relatively pure preparations. The higher specific rates of oxygen uptake, the lack of residual respiration, and the lighter color of the zonal preparation observed in the present study all support that conclusion that the zonal preparation contained less contaminating

protein. Other reports appearing in the literature have consistently shown the advantages of isolating mitochondria on sucrose step gradients (27, 77).

C. Mitochondrial Oxidations

1. Oxidation of Succinate

The rates of succinate oxidations for mitochondria from 4 to 6 day old cotyledons are not greatly different from the rates reported for other mitochondrial preparations. Douce et al (27) reports that highly purified mitochondria from mung beans have succinate oxidation rates of near 400 nmoles/min.mg protein.

The lower respiratory control ratios obtained with succinate than with α-ketoglutarate or malate probably occurs because the alternate pathway is not under the control of phosphorylation. This is shown by the observation that ADP added after cyanide had no effect on the respiration rate when succinate was the substrate. It was noted that preparations that had lower cyanide resistant rates, such as preparations from 1 day old cotyledons, often had higher respiratory control ratios with succinate than regular preparations (Table IV).

The K_i for cyanide inhibition of succinate oxidation reported in this study also agrees fairly closely with figures reported in the literature. Ikuma and Bonner (50) report a value of 4 uM. As seen from the inhibition curve (Figure 5) the concentration used in routine assays (0.02 mM) was adequate to completely inhibit cytochrome oxidase. The amount of cyanide resistance in the pea cotyledons mitochondria is similar to that reported for mung bean mitochondria and for Alaska pea hypothyl mitochondria (25).

The inhibition by SHAM of the cyanide-resistant respiration indicates the operation of an alternate oxidase similar to that described in the literature. SHAM is a relatively specific inhibitor (87, 90) of the alternate oxidase in plant mitochondria with a slightly lower inhibition constant than m-CLAM. However it was used in this study because it is commercially available while m-CLAM is not. The half maximal inhibition constant for SHAM has been reported to be 0.06 mM with mung bean mitochondria as compared with 0.17 mM found in this study.

Inhibition of state 4 respiration and not state 3 (Figure 6) respiration supports the general contention that the cyanide-resistant pathway operates when there is an excess of electrons that the cytochrome pathway cannot handle. It has been reported (2, 3) that in mung beans the alternate pathway is maximally active during state 4 but is not engaged at all in state 3. The results reported in the present study (Figure 8) differ somewhat from this. However the methodology used to determine this information is not that reliable. Small errors in the drawing of the lines on the graphs could change the results considerably.

2. Oxidation of α-Ketoglutarate

The oxidation rate of α-ketoglutarate of near 130 nmoles O_2/min.mg protein from mitochondria from 4 day old cotyledons (Figure 9) is somewhat slower than the 220 nmole rate reported for purified mung bean mitochondria (77). However the difference is not large and can perhaps be accounted for by differences in the methods used to measure protein.

The fact that respiratory control was possible even in the presence of cyanide indicates that phosporylation is associated with the alternate pathway. The ADP:O ratio of near 2 supports the contention that the

branch point is between phosphorylation sites 1 and 2 of the respiratory chain.

Although the percentage of the respiration resistant to cyanide was much greater for succinate than for α-ketoglutarate, the rates of cyanide resistant respiration in terms of nmoles O_2/min.mg protein were roughly the same for both substrates. This could indicate that electrons from both substrate dehdyrogenases had equal access to the alternate pathway. Other authors have reported greater cyanide resistance with succinate than with malate and have concluded that the cyanide resistant pathway is more available to the dehydrogenases of succinate that to the dehydrogenases of other substrates. This may be true for other tissues but does not seem to be true for pea cotyledon mitochondria.

3. Oxidation of Malate

The oxidation of malate (140 nmoles O_2/min.mg protein) (Figure 10) is considerably lower than that reported for mung bean mitochondria (450 nmoles O_2/min.mg protein) (27). However the mung bean study used 30 mM malate while 10 mM malate was used in this study. It is quite likely that the 10 mM concentration favors the operation of malate dehydrogenases whereas malic enzyme is probably also operating at 30 mM malate (109). This may account for the lower rate of malate oxidation by the pea mitochondria as compared with the mung bean mitochondria, or it may simply be that the capacity of the malate dehydrogenases is lower in the pea cotyledon mitochondria.

It has frequently been observed that plant mitochondria exhibit a more rapid state 3 rate after 2 or 3 cycles of ADP additions. It is

felt that the lower initial state 3 rate may be limited by the intramitochondrial substrate concentration (49). Malate oxidation does not behave simiarily, perhaps because of a build-up of oxaloacetate (110). A two phase state 4 rate has been interpreted by Palmer (79) in terms of kinetic compartmentation. He feels that the second phase may be due to a transfer of electrons to the nonphosphorylating NADH dehydrogenase. The experiments in this study add little to the above observations other than confirming that mitochondria from pea cotyledons behave similarily to other plant mitochondria. No reason was found for the low malate oxidation rate observed in some preparations.

Access to the alternate pathway seemed as equally available to malate oxidizing enzymes as to the other Krebs cycle dehydrogenases. Points concerning alternate pathway phosphorylation and the branch point mentioned in the discussion of α-ketoglutarate oxidation, apply to malate oxidation as well.

4. Oxidation of NADH

Although the oxidation of exogenous NADH was very rapid (Figure 11) it seemed to have limited access to the alternate pathway. This would be difficult to explain if ubiquinone were the branch point and if the oxidation of exogenous NADH proceeded through ubiquinone. However the point at which electrons from the externally added NADH enter the respiratory chain is not definitely known. Von Jagow and Bohrer (104) have shown that cyanide-resistant duroquinol oxidation in Neurospora proceeds backwards from a component in Complex III to ubiquinone and then to the alternate pathway. The same situation may be occurring with NADH oxidation in the pea cotyledon mitochondria and this would account

for the limited access to alternate pathway by electrons from NADH oxidation.

5. Oxidation of Citrate

Since citrate oxidation does not yield NADH directly, the reducing equivalents received by the respiratory chain must have come from the oxidation of isocitrate, which would account for the slowness of the respiration rate (Figure 12). This experiment is important since it once more shows that all of the Krebs cycle intermediates tested had equal access to the alternate pathway. One could speculate that if the function of the alternate pathway was to allow conversion of one of Krebs cycle intermediates to another in the presence of a high energy charge, the amount of cyanide-resistant respiration might be influenced by which intermediate was present. This did not appear to be the case.

6. Oxidation of Ascorbate-TMPD

The electrons from the oxidation of an ascorbate-TMPD mixture are passed directly to the cytochrome oxidase region of the electron transport chain (7). Therefore complete inhibition of the oxidation of ascorbate -TMPD by cyanide would be expected. This did not occur (Figure 13). The remaining respiration can perhaps be accounted for by residual cytochrome oxidase activity or perhaps by a slow auto-oxidation of the ascrobate-TMPD mixture. Since the addition of succinate to the cyanide-inhibited state caused an acceleration of the respiration rate and since this rate was inhibited by SHAM (Figure 13), it is clear that residual cytochrome oxidase activity is not great enough to account for the cyanide-resistance observed in the pea cotyledon mitochondria. This

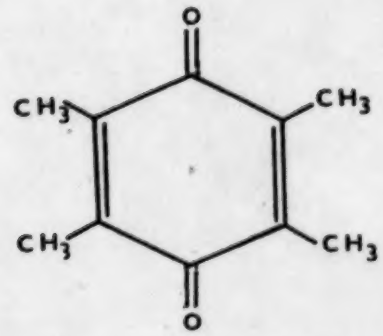
experiment is essentially a repeat of the earlier Wiskish and Bonner experiment (110), which showed that cyanide-resistant respiration could not be explained by residual cytochrome oxidase activity.

A small amount of reverse electron transport from the ascorbate-TMPD to the alternate oxidase may also have been occurring. The experiment in which ATP was added after the cyanide was designed to test this hypothesis. ATP is necessary for reverse electron transport to occur (7). The results can be interpreted as meaning it was. However too much emphasis cannot be placed on these results as the measured rates are very slow and bordering on the limits of sensitivity of the oxygen monitor.

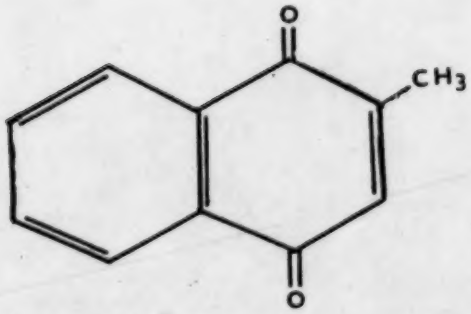
7. Oxidation of Duroquinol

Duroquinol closely resembles Coenzyme Q in structure (Figure 27). In its reduced form it is believed to react near the same site as reduced Coenzyme Q (25). Duroquinol oxidation proceeds without going through endogenous ubiquinone but there is some evidence to indicate that high duroquinol concentrations can cause a reduction of the ubiquinone in the mitochondria.

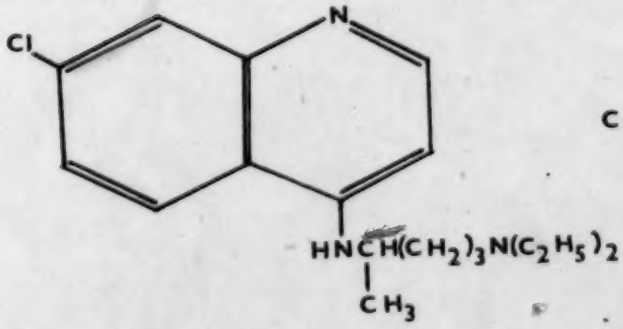
It is interesting to speculate why an analog such as this does not readily donate electrons to the alternate path (Figure 14). The most likely reason is that the redox potential of the duroquinol (E^{o} = + 35 mv pH 6.0) is higher than that of the first member of the alternate pathway. The fact that duroquinol does not readily donate electrons to the alternate pathway does provide some evidence that ubiquinone is the branch point. If it is assumed that duroquinol reacts at the same site as the reduced ubiquinone, then the branch point must be prior to the site of ubiquinone oxidation, either at ubiquinone or prior to it. It is



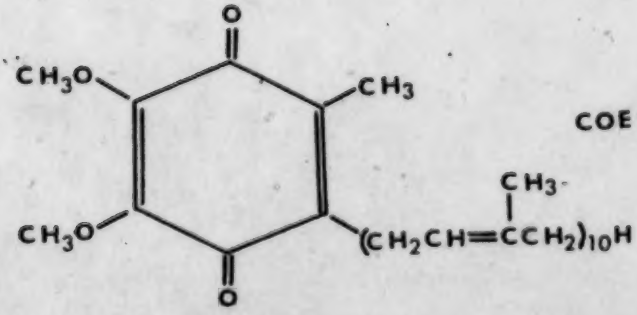
DUROQUINONE



MENADIONE



CHLOROQUINE



COENZYME Q_{10}

FIGURE 27. The chemical structures of menadione, duroquinone, chloroquine, and Coenzyme Q_{10}.

unlikely that the branch point is prior to the ubiquinone site, since in the pea cotyledon mitochondria electrons from endogenous NADH and from succinate have equal access to the alternate pathway.

It is posssible that a small amoumt of the duroquinol oxidation was proceeding by the alternate pathway (Figure 14). Since SHAM increased the autoxidation rate of duroquinol and therefore the exact amount of alternate pathway activity could not be determined. However it must have been very low since greater than 90% of the oxidation was cyanide sensitive and small amount of the remaining respiration probably arose from a slow auto-oxidation of duroquinol.

8. Oxidation of Menadiol

Menadiol oxidation also takes place through the sites at which ubiquinone is oxidized (25). However part of the oxidation of menadiol also proceeds through ubiquinone since menadiol has a lower redox potential than duroquinol (E^{o} = -1mv). Menadiol therefore would seem to be a good tool for determining whether ubiquinone is involved in alterate pathway oxidations. However the extremely rapid auto-oxidation of menadiol (Figure 15) coupled with the problems associated with the use of SHAM, make it difficult to make concrete conclusions. Although it was found (Figure 15) that cyanide resistant rate was greater than the initial auto-oxidation rate, this does not necessarily mean that the electrons were going to the alternate pathway. It may have simply been a diaphorase-like activity.

Bonner and Rich (18) use the SHAM-sensitive menadiol:O_2 reductase activity as the assay technique in their experiments where they claim to have solubilized the alternate oxidase. They do not report any of the

interactions found in this study, and do not give any details of their assay procedure.

9. Inhibition by Rotenone

The malate oxidation of the pea cotyledon mitochondria was quite resistant to rotenone (Figure 16). In fact it is somewhat greater than that reported for other plant tissues. However rotenone insensitivity seems to vary a greal deal depending on the tissue used (111).

The rotenone and cyanide experiments are interesting since they provide evidence against there being two parallel respiratory chains. Lips and Biale (66) proposed this hypothesis on the basis of their work with tissue slices in which the respiration was resistant both to cyanide and amytal. Since in the pea cotyledons the cyanide resistant respiration was not as great in the presence of rotenone as its absence, but was still present to a fairly large degree, it would seem likely that the cyanide-resistant pathway was receiving electrons from both rotenone sensitive and insensitive dehydrogenases.

10. Inhibition by Chloroquine

Inhibition of cyanide-resistant respiration by chlorquine has not been shown before. It is the first time that a ubiquinone analog has unambiguously inhibited cyanide-resistant respiration. Dibromothymoquinone has been used to inhibit the alternate oxidase but part of its inhibition may simply result from a non-specific effect on membrane fluidity (91).

Chloroquine is a unqiue inhibitor in that it inhibits the internal NADH oxidizing system, but it does not inhibit the succinate oxidizing

system (25). It has also been shown to uncouple phosynthetic phosphorylation (46). All these observations have been confirmed in this study. Experiments were done at two pHs since at pH 7.2 chloroquine does not pass through the inner mitochondrial membrane. For that reason most previous experiments using chloroquine have been conducted with inverted (inside out) electron transport particles. About 60% inhibition of the NADH oxidation of these particles can be obtained with a 10 uM concentration of chloroquine (25). Crane (25) also reports that there is another inhibition site in right side out particles at 100 uM chloroquine. No evidence for this site was found using whole pea cotyledon mitochondria. The inhibition by chloroquine raises some interesting questions concerning the location of the alternate oxidase. Since inhibition of the alternate oxidase occurred at pH 7.2, it would appear that the alternate oxidase must be located on the outside of the inner membrane. However some uncoupling of respiration took place at pH 7.2 (Figure 18) indicating perhaps that a small amount of chloroquine was passing through the inner membrane. Also since relatively high concentrations of chloroquine were required to inhibit the alternate oxidase, one could argue that the inhibition of the alternate oxidase was being carried out by a small amount of chloroquine passing through the inner membrane. However since malate oxidation was unaffected at pH 7.2 by chloroquine but was dramatically affected at pH 8.2 it seems unlikely that chloroquine was passing through the inner membrane. An experiment conducted to compare the chloroquine inhibition of succinate-supported cyanide resistant respiration at pH 7.2 and 8.2 was complicated by the fact that the alternate oxidase itself was considerably inhibited at pH 8.2. No evidence could be found though for greater inhibition of the

alternate pathway by chloroquine at this pH. Therefore it seems likely that the alternate oxidase is located on the external surface of the inner membrane. The structural similarity of chloroquine to ubiquinone (Figure 27), and the previously reported (25) mode of action of chloroquine on beef-heart submitochondrial particles, favor the idea that chloroquine inhibits the alternate oxidase by competing with reduced ubinquinone for an enzyme site.

D. The Redox State of Coenzyme Q

The experiments measuring the redox state of Coenzyme Q (Table II) do not yield a great deal of information. In all the experiments where SHAM was present the ubiquinone was in a more oxidized state than when it was absent. There are three possible explanations for this. The first is that a small amount of SHAM, which absorbs strongly in the ultraviolet region, was carried with the ubiquinone through the extraction procedure. The possibility is unlikely since control experiments showed that SHAM was virtually insoluble in petroleum ether and partioned rapidly into the methanol-water phase. The second explanation is that the SHAM chemically oxidized the ubiquinone during the extraction procedure. This idea is negated somewhat by the fact that the SHAM rapidly partioned into the methanol-water phase. However, it is supported by the experiments with SHAM and menadiol. The third option, that the in vivo effect of SHAM was to inhibit the reduction of ubiquinol is even more likely. If this were the case then the branch point would have to be located prior to the ubiquinone and there would have to be a separate pool of ubiquinone, incapable of interacting with the main pool of ubiquinone, for the alternate pathway. The ubiquinone in animal

mitochondria at least is generally considered to be a homogenous pool (25). Therefore it was felt that the results from the experiments in which SHAM was used were artifactual.

The main piece of information that can be gleaned from these experiments then is the oxidation state of the ubiquinone under state 3 and 4 conditions. The values obtained of 29% reduction in state 3 and 52% reduction in state 4 are in line with values obtained by Storey (98) using spectrophotometric methods. This provides an independent confirmation of his results and indirectly of his conclusions concerning the role of ubiquinone in the alternate pathway. He believes that these values satisfy the requirements placed on the branch point by the work of Bahr and Bonner (2, 3), and therefore ubiquinone is likely the alternate oxidase.

E. Ubiquinone Extraction Experiments

Because of the fragility of the alternate pathway, no definite conclusions concerning the alternate pathway can be made from these experiments. Von Jagow and Bohrer (104) obtained similar results with mitochondria from chloramphenicol treated Neurospora. However, in their case they started with considerably more cyanide resistance and some of this did survive the lyophilization, extraction, and reincorporation procedures.

Nonetheless the extraction and reincorporation experiments are interesting because since they do say something about ubiquinone function in the mitochondria and do allow some comparisons to be made between plant and animal mitochondria.

It has been observed previously that partial ubiquinone extraction does not affect all oxidizing systems to the same extent (25). In beef heart mitochondria succinoxidase is lost first, while in brain mitochondria NADH oxidizing ability is lost first. The plant mitochondria behave more like the brain mitochondria in this case. These observations have been interpreted as showing structural differentiation of the ubiquinone pool with the mitochondrial membrane.

Duroquinone does not restore mitochondrial activity in pentane extracted beef heart mitochondria. Duroquinone can be reduced by the NADH dehydrogenase only if ubiquinone is present, and the activity is rotenone sensitive (25). Since in the present study duroquinone restored NADH oxidizing activity but not succinate oxidizing activity, it is possible to speculate that the existence of a rotenone insensitive dehydrogenase in plant mitochondria could explain this difference between the plant and animal mitochondria. The rotenone insensitive oxidase may be able to interact directly with duroquinone without the intervention of Coenzyme Q.

F. The Effect of Cotyledon Age on Mitochondrial Activities

One of the aims of this study was to study some factors that could conceivably control the amount of cyanide-resistant respiration with the hope of gaining insight into the physiological role of the alternate pathway. In this regard pea cotyledons provide an interesting system to work with since they undergo a number of striking developmental changes in a relatively short period of time. These developmental changes extend to the mitochondria.

It is generally believed that the mitochondria present in the dry seeds are relatively disorganized, but that they develop rapidly to supply the energy needs of the cotyledons (4, 5, 75, 95). This is reflected in the present study by the low rates of oxygen uptake and relatively poor respiratory control obtained after 4 hours of imbibition (Table IV). The rapid development is illustrated by the fairly rapid succinate oxidation and good respiratory control obtained after 24 hours of imbibition. The fact that succinate oxidizing ability develops more rapidly than activity with other Krebs cycle intermediates is interesting. It may reflect the importance of the glyoxalate pathway during the early stages of germination. Earlier it was mentioned that succinate is oxidized to malate by the mitochondria when this pathway is operating, but that further mitochondrial oxidations are inhibited. It is not known how this is achieved but the results of the present study suggest that it may be possible because of relatively low concentrations of the enzymes responsible for malate breakdown.

Oxidative activites in the mitochondria continue to increase until about the fifth or sixth day of germination (Table IV). This probably reflects an increasing energy demand by the cotyledons and the growing axis. It has been found that radicle emergence can occur without any of the cotyledonary reserves (4,5). This would mean that reserves are starting to be transferred to the axis about the time that the mitochondria are beginning to assume full oxidative activities (after the second day of germination).

After the sixth day of germination the oxidative activities begin to fall off. This reflects the senescence of the cotyledons. It

became increasingly difficult to isolate mitochondria from these cotyledons. The yields of mitochondria were lower and many of the cotyledons were infected with disease. It is quite probable however that the greatest transfer of reserve material from the cotyledons to the axis occurs just before the onset of, and during this phase of development (5).

The development of cyanide-resistant respiration roughly parallels that of other respiratory activities. This is shown in Figure 28, which compares the development of two enzymatic activites as determined by Solomos et al (95), and the development of cyanide resistant respiration. This pattern of development has also been found in similar studies done on chick peas (21). It is in contradiction with another view (114) put forward which held that cyanide-resistant respiration operated very early in the germination of several seeds. The latter study did not utilize isolated mitochondria and the results may reflect the involvement of non-mitochondrial oxidative pathways.

It is interesting that at the very early stages of germination there is no cyanide resistance. This situation extends until after the first 24 hours of germination even though mitochondria from one day old cotyledons are able to oxidize succinate rapidly. It is not known why this situation exists. It may reflect the poor development of mitochondrial membranes. A recent study has emphasized the importance of mitochondrial membranes in cyanide resistant respiration (107, 108).

The fact that the greatest amount of cyanide-resistant respiration occurs when the mitochondria have high oxidative activities, and when the transfer of reserves from the mitochondria is the greatest is probably significant. It is consistent with the ideas of Palmer (77)

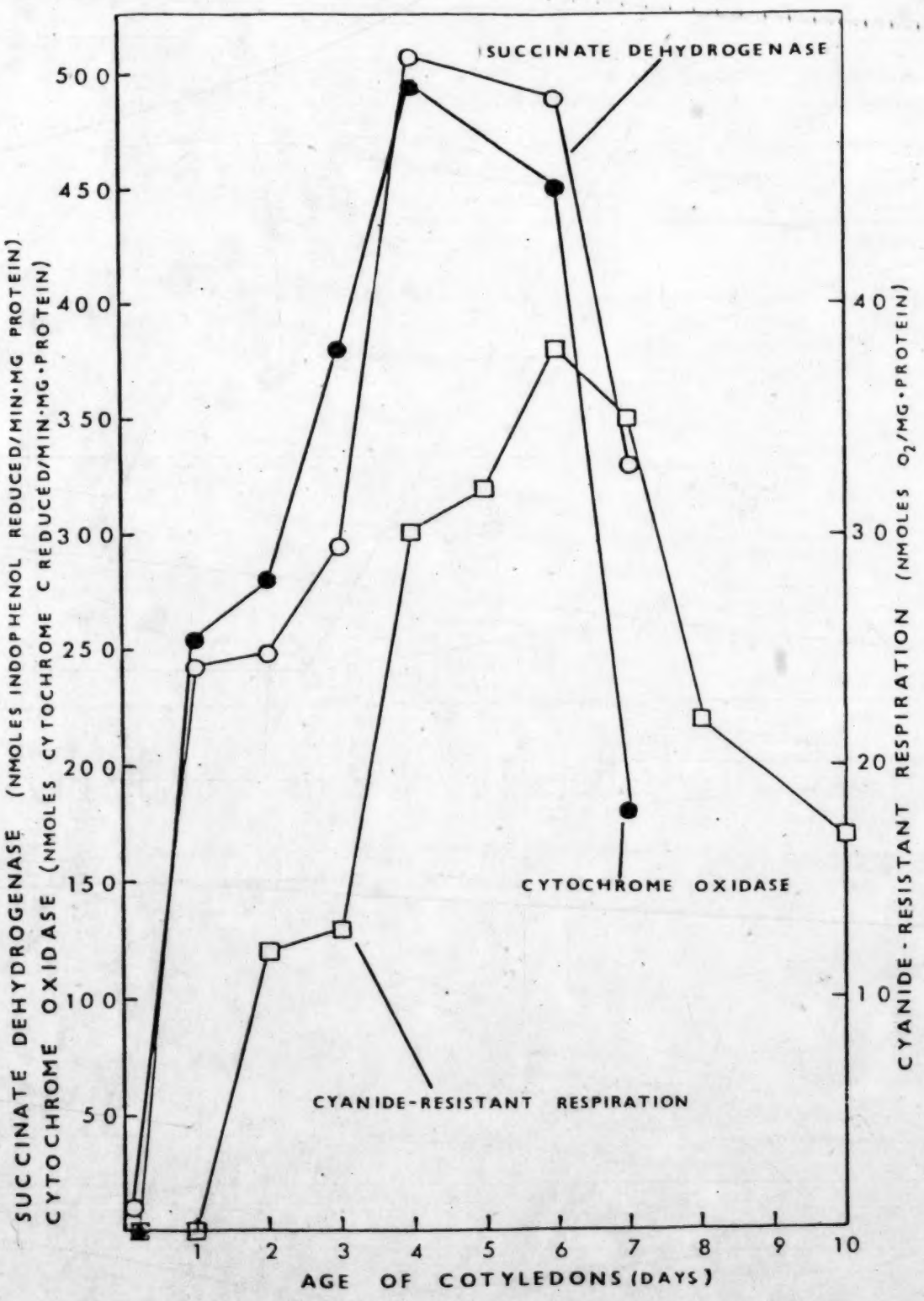

SUCCINATE DEHYDROGENASE
CYTOCHROME OXIDASE
CYANIDE-RESISTANT RESPIRATION
SUCCINATE DEHYDROGENASE (NMOLES INDOPHENOL REDUCED/MIN·MG PROTEIN)
CYTOCHROME OXIDASE (NMOLES CYTOCHROME C REDUCED/MIN·MG·PROTEIN)
CYANIDE-RESISTANT RESPIRATION (NMOLES O2/MG·PROTEIN)
AGE OF COTYLEDONS (DAYS)
500
450
400
350
300
250
200
150
100
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10

FIGURE 28. Changes during germination in the activity of succinate dehydrogenase, cytochrome oxidase and cyanide-resistant respiration.

The data for cyanide-resistant respiration is taken from Table IV with succinate as the substrate. The data for cytochrome oxidase and succinate dehydrogenase is taken Solomos et al (95).

concerning the function of the alternate pathway. The alternate pathway may be involved in the interconversion of organic acids in the presence of a high energy charge. Krebs cycle intermediates are required for the synthesis of amides, which are one of the forms by which reserves are transferred from the cotyledons.

It is interesting that the amount of cyanide resistance does not increase during the senescence of the cotyledons. Other workers have associated the cyanide-resistant pathway with senescence (45). It has been observed that the amount of cyanide-resistant respiration increases as ageing proceeds. However most of these studies have used whole tissues rather than isolated mitochondria. It may be that the capacity of the alternate pathway does not increase during senescence, but rather electrons tend to pass to the alternate pathway rather than to the cytochrome pathway because the energy requirements of an older tissue are not as high. The control mechanisms (31) proposed for the alternate pathway in which electrons are shunted to the alternate pathway in the absence of ADP, allows this to occur.

G. Alterations of the Germination Environment

1. Inclusion of Chloramphenicol

The results reported in this study differ from those obtained with Neurospora, and with rice coeloptiles. It may be argued that the chloramphenicol was not getting to the site of action since mitochondrial activity was not greatly affected. However growth of the chloramphenicol-grown peas was slower indicating that the chloramphenical was having some effect on the peas. Also the concentration in this study was twice that used in the study done with rice. The discrepancy may

reflect species differences between rice and peas. There are some experimental results (70, 71, 89) that might indicate different factors control cyanide resistant respiration in dicotyledons than in monocotyledons. However this hypothesis has not been tested. It should be pointed out that rice is exceptional in that it can also germinate in the absence of oxygen, something very unusual for plant seeds. However, for the development of cyanide resistant respiration in rice, oxygen was required (70).

2. Effect of Azide

In many micro-organisms, inclusion of respiratory inhibitors in the incubation medium leads to the development of the alternate pathway. Being an inhibitor of cytochrome oxidase, azide might be expected to have the same effect. However cyanide resistance was not promoted in peas. Nevertheless mitochondrial respiratory characteristics were affected. This is not surprising considering that many enzymes that azide affects. Azide was used in these experiments because of the volatility of cyanide made interpretation of experiments conducted with it difficult.

3. Effect of Light

There is no a priori reason for expecting light to influence the cyanide resistant pathway. However it is remotely possible that light might affect the development of the cotyledons by making the young seedling self-sufficient earlier. Hower this did not appear to be the case. It is not known how significant the decreases in the respiratory control ratio were since the effect was not investigated intensively.

4. Effect of Ethylene

The changes in cyanide-resistant respiration in response to ethylene reported in this study, including the synergistic effect of oxygen, closely resemble the responses report for potato tubers (89). Initially it was not clear if the response was a direct effect of the ethylene or was an indirect effect occurring as a result of the growth inhibition caused by ethylene. However, the experiment in which cotyledons alone were treated with ethylene would seem to indicate that the response was a direct effect of the ethylene. It is not known how this is achieved or why oxygen would be synergistic. However it has been reported elsewhere that high oxygen tensions promote synthesis of the alternate oxidase (71, 89).

V. SUMMARY AND CONCLUSIONS

The preceding experiments have shown that an alternate, cyanide-resistant respiratory pathway operates in the mitochondria of pea cotyledons. The following evidence supports this conclusion: 1) there is incomplete inhibition by cyanide of the respiration of the pea cotyledon mitochondria, and 2) the remaining respiration is inhibited by SHAM. Residual cytochrome oxidase activity, either as a result of incomplete inhibition by cyanide or slow turnover of the inhibited form of cytochrome oxidase cannot account for the cyanide resistant respiration. The use of the zonal procedure for the isolation of mitochondria insures that the location of the cyanide-resistant respiration is intra-mitochondrial.

The relationship between the alternate and main respiratory pathways has also been examined to some extent. All Krebs cycle intermediates tested were able to donate electrons to the alternate oxidase. However only a small part of the oxidation of exogenous NADH proceeded through the alternate pathway. These experiments indicate that the alternate pathway in pea cotyledons branches from the main respiratory pathway between the first and second sites of phosphorylation. It was also found that the cyanide-resistant respiration was inhibited somewhat by rotenone, indicating that there was not a rotenone-insensitive, cyanide-insensitive, non-phosphorylating pathway operating in parallel with the main respiratory pathway.

A previously unreported inhibitor of cyanide-resistant respiration, chloroquine was found. Inhibition by chloroquine indicates that ubiquinone is probably involved in the cyanide-resistant pathway and points to a location outside the inner mitochondria membrane as the site for the alternate oxidase. Unfortunately the involvement of ubiquinone in cyanide-

resistant respiration could not be confirmed by ubiquinone extraction and reincorporation experiments. However these experiments do point out some similarities and differences between plant and animal mitochondria.

Since duroquinol oxidation did not proceed through the alternate pathway, although duroquinol oxidation resembled ubiquinol oxidation in other ways, it would appear that branching to the alternate pathway must occur just prior to the ubiquinol oxidation sites of the main respiratory chain, namely at ubiquinone.

This study has also shown how the cyanide-resistant respiration in the pea cotyledons varies with the development of the pea seedling. It is absent at the initial stages of germination, at a time when the inner cristae membranes are not well developed, but is at its maximum near the sixth day of germination. It is possible that the alternate pathway is playing a role in the mobilization of reserves from the cotyledons, since this process is also maximally active at this time. It may be allowing certain mitochondrial reactions, such as the interconversion of organic acids, to occur at a time when the energy demands of the cotyledons may not be as great.

In addition this study has determined the effect of some external environmental factors on the development of cyanide-resistant respiration in pea cotyledons. It has been shown that the presence of light did not affect the amount of cyanide resistance. Neither did the inclusion of chloramphenicol or azide in the germination medium. These last two experiments do not lend support to the idea that the cyanide-resistant pathway acts as a protective mechanism when inhibitors of cytochrome oxidase are present.

It has been shown that the presence of ethylene can increase the

amount of cyanide resistance. This may indicate that the development of cyanide-resistant respiration in pea cotyledons is normally under the control of this plant hormone.

In summary, this study has shown: the presence of cyanide-resistant respiration in pea cotyledons, some relationships between the normal and cyanide-resistant respiratory pathways, the development of cyanide-resistant respiration during pea cotyledon germination, and the influence of some external factors on the development of cyanide-resistant respiration in pea cotyledons. Other aspects of respiration and the electron transport chain in plant mitochondria have also been studied and discussed.

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